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ENZYME ACTIVITY IN TERRESTRIAL SOIL IN RELATION TO EXPLORATION OF THE MARTIAN SURFACE

Professor A. D. McLaren

1 January 1971

Department of Soils and Plant Nutrition

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I. PREFACE

Our objective are to explore enzyme activities in soil, including abundance, persistence and localization of these activities, and to develope procedures for detection and assay of enzymes in soils suitable for presumptive tests for life in planetary soils.

Thus far we have developed a sensitive test for soil urease, based on hydrolysis of heat stable, ^{14}C-urea and have described the urease activity of ancient and buried soils.

We have also explored in a general way the behavior of enzymes in non-classical systems, e. g. on surfaces, in gels and coacervates, and at low humidity, as an aid to understanding enzyme action in the heterogeneous systems, soil.

At present we are working on suitable extraction procedures for soil urease and have been measuring activity in one such extract in order to study low urease is complexed in soil organic matter. We are also attempting to analyse and predict microbial growth and the kinetics of consecutive reactions in soil systems.

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We are all grateful for the able assistance of Mr. A. H. Pukite in much of the experimental work to be described.

To the Utah State University Ecology Center Project, Ecology 35, we are indebted for partial support of the research of Dr. Skujins since he left the University of California; this support enabled us to include the section on to persistence of enzyme activity in soil.

II. Vectorial Aspects of Soil Microbial Ecology: A POINT OF VIEW

Conceptually, biochemistry in soil has much in common with that of a plant or animal organism. All three are systems with both intracellular metabolisms and extracellular enzyme reactions. The extracellular reactions most often involve heterogeneous catalysis (McLaren and Packer, 1970), and in the case of soil the enzymes are mostly in an insoluble state, i.e. the enzymes are cross-linked with insoluble, colloidal organic matter (McLaren and Peterson, 1967). All three systems also exhibit vectorial reactions.

The concentration of a metabolite [S] is a function of time t and the rate of change is a function of the amount of catalyst E, the temperature, the pressure, the water activity, amounts of coreactants, surface pH, bulk pH, and the numbers of cells and cell species of the active cell population. Frequently in soil microbial experiments, to a sample of moist soil there is added a substrate at some bulk concentration [S]. The substrate becomes distributed among a number of surface and colloidal sites by adsorption and by ion exchange and the concentration can no longer be specified uniquely. Thus any model of the chemical activities of soil is at once, of necessity, a gross oversimplification. If the soil is an isolated sample, closed with respect to the further addition of S and open to air or not, one also has a "very unnatural" situation, a statement that may be justified in the following way. In such an isolated system the organisms are distributed in the system as zooglea, or

simply, in pairs, as spores etc., both in easily elutable and in tightly bound formson the mineral and humus particles. The product P of reaction of S, secreted by any microbe or mediated by extracellular enzyme action, is available by diffusion and/or by microbial movement for further chemical reaction. Customarily one expresses a rate of change of S in such a system as $- d[S]/dt = d[P]/dt$ and these are scalar quantities. For a reaction sequence such as $NH_4^+ \longrightarrow NO_2^- \longrightarrow NO_3^-$ all forms of nitrogen N_i are distributed throughout the bulk soil sample and are more or less equally available to the enzyme systems present (intra- or extracellular). That is to say, for this example, we have a heterogeneous mixture of substrates and microbes distributed within the bulk soil volume in a closed system and both Nitrosomonas sp. and Nitrobacter sp. have simultaneous and nearly equal access to N_i.

In situ, however, soils have a profile, i.e., a change of properties with depth. If NH_4^+ is supplied at the surface of the soil and a solution with concentration $[NH_4^+]$ moves downward, it can be oxidized by Nitrosomonas to NO_2^-. If at the surface $[NO_2^-]$ is small initially, Nitrobacter will be exposed to only small concentrations of NO_2^-, whereas at some depth below the surface, X, the concentration of NO_2^- will be greater and Nitrobacter will experience an increased availability of nutrient. In other words the concentrations and ratios of concentrations of NH_4^+, NO_2^- and NO_3^- will not only vary with time in a given volume element, but will vary from volume element to volume element with a downward change in X. An organism, such as Nitrobacter, lower in the soil profile will be exposed to ratios of concentrations of $[NH_4^+]/[NO_2^-]/[NO_3^-]$ differing from those in volume elements above it and the best way to express the changes of bulk

concentrations in the system is as $d[NH_4^+]/dX$, $d[NO_2^-]/dX$ and $d[NO_3^-]/dX$. (By bulk concentrations are meant the total amounts of each of N_i divided by the bulk volume element of the soil at any X). These are clearly vector quantities since they have both magnitude and direction (downward in this case). Thus soil biochemistry has the character of a vector chemistry in common with other transport situations in nature. Uptake of sugars by plant roots or transport into intestinal villi, are other examples (McLaren and Packer 1970).

Assuming that the flow rate of NH_4^+ in a soil, initially free of NH_4^+, is given by $f = \frac{X}{t}$ where X is in centimeters and t is in hours, we may write for the sequence $NH_4^+ \longrightarrow NO_2^- \longrightarrow NO_3^-$, $d[NH_4^+]/dt = f\ d[NH_4^+]/dX$ etc. A solution of equations for this reaction sequence depends on the growth, maintenance energy and waste biochemistry of the organisms involved, i.e. on the mechanisms assumed for each step in the reaction sequence. Examples of such solutions for soil profiles have been published (McLaren, 1970).

Summary

Prediction of rates of biochemical change of chemicals in soil during downward movement is an important problem, especially since the changes reflect the growth of microorganisms and vice versa. The oxidation of nitrogen compounds in a soil profile is discussed by way of illustration and a mathematical model is outlined.

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II. PERSISTENCE OF ENZYMES IN SOIL

Abstract

Microorganisms and plant roots release a variety of enzymes into soil. These enzymes exist in an active state in soils for a certain period of time. Most of the enzymes are inactivated rapidly, but some residual activity may be detected after long time periods.

Geologically preserved permafrost soils, and soils stored about 60 years in an air-dried state have been examined for urease, dehydrogenase, and phosphate activities. Measurable dehydrogenase activity may be detected in relatively fresh soils only; it is thought to reflect the rate of the overall microbial metabolism or biomass. Urease and phosphatase activities were observed in 8,715 and 9,550 years old permafrost peat samples, whereas in a 32,000 years old buried soil sample these enzymatic activities were below detectable levels. In all examined soils urease activity reflected the organic matter content. Phosphatase activity in the 60 years old desert soil samples reflected the microbial numbers and the soil type, whereas with other soils the correlation may be better with organic matter content.

Generally, microorganisms have been recovered from over 300 years old air-dry stored soil samples, and preserved seeds of similar and older ages have been germinated, indicating that enzymes may persist in their native environment at very low water activities for extended

periods of time. It is likely that enzymes, being sorbed on surfaces in such dry environments, specifically, the soil, are protected against further inactivation by the physical restrictions in sorbed state and by the osmotically and hygroscopically held water layer. Alternatively, the enzyme protein may be covalently bound to other soil organic particulate matter, and the protein molecular structure maintained.

Introduction

Most soil organisms release enzymes into the soil and many biological transformations in soil are partially or wholly catalyzed by enzymes found outside the living soil organisms (Skujiņš, 1967). In order to function these enzymes should remain in the soil outside the living cells in an active state for a certain period of time.

Upon the death of cells, collapse of cell walls and disintegration of membranes, protoplasmic constituents are released into soil. Although most of the released protein, carbohydrates, lipids, and other cellular material may be quickly metabolized by other organisms, some enzymes apparently persist in soil in an active state and appear quite resistant to denaturation in the soil environment. Almost all attempts, however, at isolating enzymes in pure form from the soil have been unsuccessful, perhaps because of the strong binding of proteins by clays and humus constituents. Consequently, such binding might be instrumental in the stability of free enzymes in soil.

In the last decades much empirical information has been collected regarding enzymatic activities in soil, but answers to a number of fundamental questions are still lacking, especially those regarding the

origins of enzymes in soils, their distribution and localization in the soil matrix, and their significance in the decomposition of soil organic matter and in humus formation. From the biogeochemical point of view it is of interest to examine the persistence of these enzymes in soils and their eventual effect on organic matter transformation, but the chemical and physical factors determining their persistance in soils are virtually unknown.

Detailed study of the activities of free enzymes in soils has encountered some fundamental difficulties. The main methodological question has been the problem of effective inhibition of microbial activity at the same time retaining the soil enzymes unaffected. It is also desirable not to disturb other chemical and physical properties of the soil. The most widely used method for this purpose has been the addition of toluene or other bacteriostatic agents to soil, another method is the high-energy radiation sterilization. The unequivocal separation of metabolic activities of microorganisms from those of extracellular enzymes in soils, however, has not yet been achieved.

Since about 1950 studies in soil enzymology have increased at a rapid rate. A new impetus to soil enzymology is being given by the recent advances in polymer chemistry, especially regarding the organic matter - protein interaction and the enzyme kinetics at surfaces and interfaces. Problems in soil enzymology have been discussed in detail recently (Skujiņš, 1967) and excellent review articles on enzymatic activities in soil have been written by Kiss (1958), Durand (1965), Hofmann and Hoffmann (1966), and by Kuprevich and Shcherbakova (1966, 1970).

Persistence of Enzymatic Activities in Several Soils

Experimental determination of longevity of enzymes in soil encounters considerable methodological and conceptual difficulties. Normally all soils have an active micro- and macroflora and fauna and the extracellular enzyme content is in a constant flux. One has to look for naturally or artificially preserved soils and to assure that during the period of preservation no influx of microorganisms has occurred and that the soil has not been disturbed by leaching or other climatological and physical factors. Two types of soils might serve for these purposes: 1) naturally preserved in permafrost, and 2) artificially preserved in soil museums and collections in sealed containers. Even with such soil samples, there exists an obvious drawback: no satisfactory "initial" control values are available.

Both types of preserved soils have been examined for some residual enzymatic activities by Skujiņš and McLaren (1968).

Soils from the Hilgard collection (located in Hilgard Hall, University of California, Berkeley) were collected in California around the turn of the century under the direction of Professors E. W. Hilgard and R. H. Loughridge (Loughridge, 1914); samples have been stored and left undisturbed since their collection. The examined soils No. 1 to No. 12 are typical non-cultivated desert and arid area samples.

The Point Barrow, Alaska samples were collected in 1964 (Brown, 1965), air dried and examined a year later; their age has been established by radio-carbon dating by the U.S. Army Cold Regions Research and Engineering Laboratory, Hanover, New Hampshire.

Dublin and Yolo soils have been stored air-dry for the period indicated. The Oxford Tract soil samples were examined fresh.

Soils are described in Table 1.

Dehydrogenase was determined according to a modified method by Kozlov (1964) (Skujiņš and McLaren, 1968), based on the oxidation of 2,3,5-triphenyltetrazolium chloride by dehydrogenase to triphenyl formazan.

Phosphatase was determined according to a method by Ramírez-Martínez and McLaren (1966a) (Skujiņš and McLaren, 1968). Na-β-napthyl phosphate is hydrolysed by phosphatases in soil and the resulting β-napthol was extracted and determined by spectrofluorophotometry.

The determination of urease activity was based on hydrolysis of ^{14}C-urea (Skujiņš and McLaren, 1969): to a 1 g soil sample in a planchet was added 10 mg ^{14}C-urea containing 10 μci ^{14}C, and 0.5 ml K-acetate, pH = 5.5, 0.05 M. The planchet was placed in a radioactive gas counting chamber and the increase of $^{14}CO_2$ in the chamber was monitored and recorded. The results were expressed as the rate of increase of counts per minute (Δ cpm/min).

Results of the determination of dehydrogenase, phosphatase, and urease activities in soils are shown in Table 2.

A measurable dehydrogenase activity was shown to exist in the fresh soils and in soils stored for few years. There was very limited or no dehydrogenase activity in the *ca.* 60 years old Hilgard Collection soils and in the Pt. Barrow soils with the exception of No. I-700; the latter reflects the high microbial activity in this sample. No other correlation between dehydrogenase activity and other factors was evident.

The assay of dehydrogenase activity in soils has been used to obtain correlative information on the biological activities of microbial population in soils. Measurable activity may be obtained without any additions of metabolites, and the results in such cases reflect endogeneous respiration (Casida *et al.*, 1964). In general, dehydrogenase activity in soil reflects the overall metabolic rate. It is likely that dehydrogenase activity would reflect the total biomass in soil, however, a verification of this assumption is desired.

Phosphatase was detected in measurable amounts in most soils; trace amounts were present in Hilgard sample No. 4, no activity was detected in Point Barrow soil No. 714.

Phosphatase activity in soils has been extensively studied but the published reports are abundant in contradictory observations and interpretations. Most of the observations show that the maximal activity occurs near a neutral pH value and not necessarily at the natural pH of the soils. In some soils that activity may increase, however, with increasing pH. Phosphatase activity usually is greater in soils with higher amounts of organic matter. This trend is also evident in most soil samples examined here. Although it appears that phosphatase activities in the stored Hilgard soils reflect the number of microorganisms, our data on the permafrost soils suggest, however, that phosphatase activity may not be directly correlated with microbial numbers in some soils. The data are consistent with previously reported findings (Ramírez - Martínez and McLaren, 1966b) indicating that most of the soil phosphatase is extracellularly bound to the soil organic matter. It is of interest to note, for example, that there was

no phosphatase (and no urease) activity in the 32,000 years old Pt. Barrow soil No. 714, although a large number of microorganisms was recovered (reflected also by the high dehydrogenase activity). It is apparent that the soil was contaminated while in transit from the source to the laboratory, as there were no specific aseptic procedures attempted during the handling of Point Barrow soil samples by the collecting agency. This sample, however, serves as an "internal control". The results indicate that the microorganisms present in this soil did not produce measurable phosphatase (nor urease) activity which were lost during the 32,000 year long burial time in permafrost.

Generalization is difficult as the organic matter rich soils as a rule also have significantly higher numbers of microorganisms. Perhaps direct correlation cannot be expected. It has been shown that phosphatase activity in soil is inversely proportional to the biologically available phosphate: adding inorganic phosphate usually diminishes the activity and even in soils having a high organic matter content it is associated with phosphate availability (*cf.* references cited by Skujiņš, 1967).

Urease activity was present in most of the examined soils, but was not detected in the 32,000 years old Pt. Barrow soil No. 714, Hilgard samples No. 2 and No. 4, and only traces in Hilgard alkali soil samples No. 9 and No. 10, also in No. 12.

Urease activity in soils appears to correlate in general with the number of microbes but its increase with increasing organic-matter content has been noted. In soil fractionation studies the highest urease activity remained associated with the clay fraction (Hoffmann,

1959). Our data indicate that the urease acitivity in preserved samples may be correlated with the organic carbon content. As stated, however, increase of microbial numbers follows the increase in organic matter.

The maximal activity of urease in most soils is found at pH = 6.5 to 7.0. In alkaline soils the activity decreases considerably, especially in carbonate-rich soils (Galstyan, 1958; Skujiņš and McLaren, 1969).

It seemed possible that urease in such stored and air-dried soils might exist in an oxidized and, therefore, inactive state. Extensive pretreatment of several preserved soils with H_2S or cysteine, however, did not increase the urease activity.

It is evident that there is some residual phosphatase and urease activity in the 8715 and 9550 years old Pt. Barrow peat and soil samples which have been subjected to permafrost for most of their established age, and also in the *ca.* 60 years old arid area soils of the Hilgard Collection. Enzymatic activities were not found in the 32,000 years old sample in spite of the bacterial contamination after collection.

The activities of purified enzymes in solution *in vitro* and of native enzymes in soil are by no means comparable with respect to the amounts of enzymes present because of the differences in physical and chemical environment. Nevertheless, some semiquantitative values regarding the enzymatic content in soils may be estimated. For example, the enzymatic activity values for one gram of Yolo soil (Table 2) are equivalent to the activity of 203 µg potato acid phosphatase (Pentex, Inc.), 0.276 µg urease (Worthington, UR), and 0.0017 µg dehydrogenase (Worthington, ADHS, estimated). The amount of protein, based on the

bacterial biomass in a fertile soil, is about 100 µg/g.

Origin of Some Soil Enzymes

The factors contributing to the total enzymatic activity in soils are:

a) proliferating microorganisms which release extracellular enzymes as a part of their physiological activity,

b) free enzymes released into soil from lysed microorganisms,

c) enzymes accessible to substrates in dead but not lysed cells,

d) free enzymes released into soil from plant roots or enzymes on the surfaces of roots,

e) any metabolic activities of live cells and roots present in the soil, and

f) contributions similar to these from soil animals.

It has been demonstrated that soil microorganisms release into soil a series of enzymes, not all of which may not be classified as typical extracellular enzymes; among them carbohydrases, transferases, lignin decomposing enzymes, and various enzymes involved in phosphate metabolism. Similarly, enzymes are released by plant roots, including invertase and phosphatases.

Many investigators have tried to correlate bacterial numbers and enzymatic activity in soil; positive correlation is rather an exception than a rule. For example, Ramírez-Martínez and McLaren (1966b) have shown that no relationship exists between soil phosphatase activity and fungal numbers. Other examples have been discussed (Skujiņš, 1967).

To approach the problem of the mechanisms involved in the origins

of enzymes in soil, high-energy radiation-sterilization methods were used. It was noted before that urease activity in soil is resistant to high-energy irradiation and its apparent activity in soil may even increase upon radiation-sterilization. A series of Puerto Rico and Hawaiian latosols were subjected to a 4 Mrad (twice the nominal sterilization dosage) and to 8 Mrad, MeV electron beam irradiation (Skujiņš and McLaren, 1969).

A dosage of 4 Mrad increased urease activity in Puerto Rico Nipe clay and in three Hawaiian soils. The activity was decreased in Wahiawa but only slightly in Lahaina soils. An 8 Mrad dose decreased urease activity in all soils, except Mahukona, below their native levels. Dublin soil was examined 7 years after irradiation; it showed a 2.6 times higher activity than nonirradiated, but otherwise identical duplicates (Figure 1).

Upon irradiation a soil urease component, most likely the intracellular microbial urease, becomes more accessible to the substrate. The increase may be caused by an unhampered diffusion of substrate and reaction products through disrupted cell membranes of dead organisms, or by urease released from disintegrating organisms. The different magnitudes of the soil urease activities at the various radiation dosages may be visualized as a result of several changes taking place in the soil during irradiation. In a nonirradiated soil the apparent urease activity may be a sum of extracellular and intracellular enzyme moieties, but, according to the method, not of vigorously proliferating organisms. Upon irradiation the extracellular moiety is inactivated at a rate $A/A_o = e^{-kD}$ (McLaren *et al.*, 1962; Skujiņš *et al.*, 1962)

whereas the intracellular moiety becomes available both to the substrate owing to disruption of cellular membranes and to radiation-inactivation similar to the extracellular moiety. It would appear, for example, that most of the urease activity in nonirradiated Nipe clay is intracellular, whereas in Lahaina clay most of the urease activity may be an extracellular accumulation.

Changes in urease activity in soils during a prolonged air-dry storage (Table 3) may be described by a similar scheme.

State of Enzymes in Soil

The questions regarding the physical and chemical state of enzyme proteins in soil are the most fascinating in the field of soil enzymology. Although some aspects of protein-soil constituent interactions have been studied in a considerable detail, especially the clay-protein sorption, there is very little information available to draw any conclusions on the aspects of state of enzymes in soil and, consequently, on the factors contributing to their stability.

Presently two types of interactions, important in enzyme stabilization in soils may be visualized: 1) protein-soil inorganic matter (esp. clay) sorptive interactions, and 2) protein-soil organic matter interactions either by sorptive or chemical binding mechanisms.

Generally, the adsorption of proteins on clays occurs in a wide pH range, and rather stable clay-protein (i.e., enzyme) complexes are formed. On such complexes proteins also may be adsorbed and hydrolyzed by previously sorbed proteolytic enzmyes. The enzymes may be desorbed with a minimal loss in activity. It is apparent that in certain cases

enzyme sorption on clays imparts stability on the enzyme (*cf.* references cited by Skujiņš, 1967, and McLaren, 1970) and it might be a factor in the accumulation of enzymatic activities in soils.

In a study on esterase activity in soil, Haig (1955) fractionated fine sandy loam: the clay fraction had the highest esterase activity, but very little in silt and sand. A similar fractionation of several enzymes was performed by Hoffmann (1959). He found that a carbohydrase activity was highest in the silt fraction; that of urease, in clay; since the number of microorganisms in the clay fraction was negligible, it was evident that urease had been adsorbed and remained active on the clay. Recently El-Sayed and McLaren (1970) have shown, however, that urease activity is associated with the soil organic fraction and as such may be separated from clays and other inorganic constituents.

Soil constituents - the organic and inorganic colloids and clay particles are always covered with a layer of water. In an "air dry" soil such water is held by a force of more than 15 atm. negative suction and may extend to distances of 100 Å and more from particle surfaces. Some properties of this "osmotically held" water (Low, 1961) suggest the characteristics of "polywater". Such a layer of water on clay surface could act as a protective agent for sorbed proteins, i.e., enzymes.

Excellent survival of microorgansims in air-dry soils is a well known phenomenon. Survival of enzymatic activities in soils has been studied, however, very little. It may be noted that the physical and chemical molecular environment, within certain limitations, might be quite similar for a protein molecule in an "air-dried" organism within the cellular reticulum and on a surface of a colloidal particle in an

"air-dried" soil; thus certain parallels on the survival of biological activity may be drawn. Incidentally, Sneath (1962) suggests that after the initial die-off of organisms in air-dried soils during the first 50 years, the "half-life" for further microbial decrease is 15 years; a ton of soil should contain a few viable organisms even after 1000 years.

In the determination of the enzymatic activities in soil, the effect of soil moisture content has been considered negligible by the earlier investigators. Scheffer and Twachtmann (1953) pointed out that invertase activity in soil decreased noticeably during the first days of air-drying, but that it became stabilized thereafter. Latypova and Kurbatov (1961) indicated that air-drying of soil reduced catalase activity to about 50% of the activity in a moist state, and Ross (1965) found that invertase and amylase activities were lowered by more than 20 percent and 50 percent respectively in air-dried soils at 20°C; in some naturally arid soils the decrease was fractional. These reductions in activities resulted mainly from the initial drying which also reduced the numbers of viable bacteria. Invertase activity in soils decreased on storage at -20°C but the changes were slight over long periods; inactivation of amylase activity was greater and increased with prolonged storage (Ross, 1965). We have observed (Ramírez-Martínez, Skujiņš and McLaren, unpublished results) that phosphatase activity in fresh greenhouse soil was reduced to 90% at 7% moisture content and to 60-70% at equilibrated air-dried conditions.

It is of interest to note that similar to enzymes and bacteria in soils, many plant seeds are known to survive for a hundred years or more in an air-dried state. It is likely that in structural systems (soil

interfaces, cellular reticulum) thin, "osmotically" or "hygroscopically" bound water layers act as structural stabilizers for protein moieties of enzymes.

The study of water-insoluble, immobilized derivatives of biologically active enzymes has received a considerable attention in the last decade. It is most attractive to consider these derivatives as models for possible analogous immobilization processes of enzymes in soil. There are three principal methods for the preparation of immobilized enzymes (Goldstein and Katchalski, 1968) of interest to soil biochemistry, yet all these methods are mild enough not to denature the protein, rather to maintain their biochemical activities:

1) inclusion of protein into gel lattices, the pores of which are too small to allow the escape of entrapped protein (the gels used here are acrylamide and starch),

2) covalent binding of proteins to a macromolecular carrier by functional groups non-essential to the enzymatic activity, and

3) covalent cross-linking of the protein by an appropriate bifunctional reagent.

One of the earlier methods involved binding of proteins to carboxymethylcellulose via the corresponding azide. Later synthetic copolymer of p-aminophenylalanine and leucine has been used extensively next to Sephadex derivatives, among others.

Many of these carriers are closely related to natural substances in soil. There have been no experiments as yet to examine if similar reactions take place in soils between soil humus polymers and enzymes. The field is wide open.

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TABLE 1

Description of soils (Skujiņš and McLaren, 1968, 1969)

Soil	Description
Hilgard No. 1	Coachella Valley, loam, surface 30 cm.
Hilgard No. 2	Same as No. 1, 60-90 cm deep
Hilgard No. 3	Victorville-Mojave River Mesa, sandy, surface 30 cm
Hilgard No. 4	Same as No. 3, 60-90 cm deep
Hilgard No. 5	Bishop-Owens River Valley, sandy, surface 30 cm
Hilgard No. 6	Same as No. 5, 60-90 cm deep
Hilgard No. 7	Bakersfield-Kern River Delta, loam, surface 30 cm
Hilgard No. 8	Same as No. 7, 60-90 cm deep
Hilgard No. 9	Tulare Experiment Station,sandy, alkaline, surface 30 cm
Hilgard No. 10	Same as No. 9, 60-90 cm deep
Hilgard No. 11	San Bernardino-Victoria Tract, sandy loam, surface 30 cm
Hilgard No. 12	Same as No. 11, 60-90 cm deep
Pt. Barrow, No. I-1182	Alaska, peat, 8715 ± 250 years old, 45 cm deep in permafrost
Pt. Barrow, No. 4	Alaska, loam, overlaying No. I-1182, 5-30 cm deep, subject to freeze-thaw cycle
Pt. Barrow, No. 714	Alaska, humic sandy silt, 5.50 m deep in permafrost, approx. 32,000 yrs. old
Pt. Barrow, No. I-700	Alaska, peat, 1.40 m deep in permafrost, 9550 ± 240 years old
Kawaihae	Island of Hawaii, Red Desert latosol loam, top 5 cm

TABLE 1 (cont.)

Soil	Description
Lahaina	Island of Oahu, latosol clay, top 25 cm
Mahukona	Island of Hawaii, latosol silty clay loam, top 15 cm
Molokai	Island of Oahu, latosol clay, top 25 cm
Wahiawa	Island of Oahu, latosol clay, top 25 cm
Nipe I	Puerto Rico, latosol clay, top 2·5 cm
Nipe II	Same as Nipe I, 15-25 cm deep
Control Soils:	
Dublin	California, adobe clay loam, stored 12 yrs.
Yolo	California, silt loam, stored 6 yrs.
Oxford Tract	Berkeley, California, loam, fresh

TABLE 2

Enzymatic activities in soils (Skujiņš and McLaren, 1968)

	Dehydrogenase H^+ µm/g/hr.	Phosphatase β-naphthol µm/g/hr.	Urease CO_2 µm/g/hr.	Number of Microorganisms[a] (10^6/g)	pH[b]	Organic C[c] percent
Hilgard 1	traces	0.11	0.005	0.83	7.9	0.40
Hilgard 2	none	0.09	none	0.059	8.0	traces
Hilgard 3	none	0.02	0.025	0.038	8.1	0.16
Hilgard 4	none	traces	none	0.011	8.3	0.19
Hilgard 5	traces	0.12	0.063	0.53	7.6	0.56
Hilgard 6	traces	0.07	0.017	0.030	8.3	0.11
Hilgard 7	0.0010	0.22	0.174	5.0	6.8	1.49
Hilgard 8	traces	0.13	0.038	1.8	7.2	0.27
Hilgard 9	0.0012	0.13	traces	0.39	9.9	0.50
Hilgard 10	traces	0.04	traces	0.16	8.6	0.50
Hilgard 11	traces	0.20	0.049	0.36	7.6	0.42
Hilgard 12	traces	0.09	traces	0.16	7.8	0.23
Point Barrow I-1182	0.0013	0.84	0.084	0.0022	6.2	19.06
Point Barrow 4	none	0.27	0.023	<0.0001	4.6	1.27
Point Barrow 714	0.0008	none	none	0.0014	7.2	3.33
Point Barrow I-700	0.0035	1.20	0.793	34	6.3	24.0
Dublin	0.0027	1.35	1.156	2.2	5.7	2.75
Yolo	0.0031	0.29	0.234	1.4	7.3	0.87
Oxford Tract	0.0040	0.66	0.258	40	5.8	1.80

a) The numbers of microorganisms (bacteria and streptomycetes) were determined by plating on yeast extract - soil extract agar. Hilgard collection samples were plated on trypticase-soy agar.

b) The pH values of the soil samples were measured in a 1:1 water suspension.

c) The organic carbon content is the difference between the total CO_2 obtained by a dry combustion method and the amount of carbonate-CO_2.

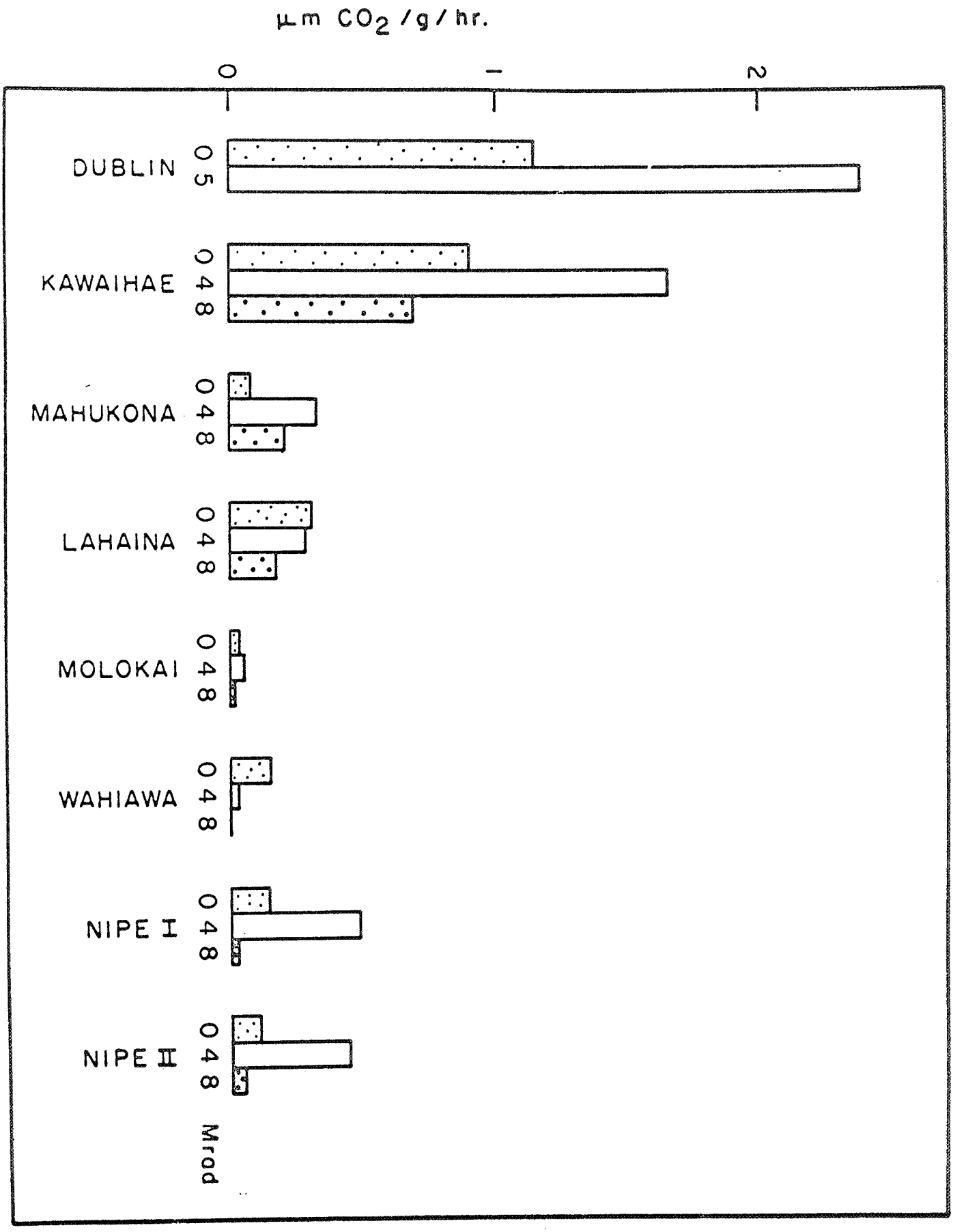
TABLE 3

Changes in urease activity during air-dry storage at 22°C (Skujiņš and McLaren, 1969)

Soil	Urease activity (CO_2 μm/g/hr)		Change in Activity, percent
	Examined July, 1965	Examined September, 1967	
Puerto Rico Soils			
Nipe I	0.292	0.144	-51
Nipe II	0.312	0.113	-64
Hawaiian Soils			
Kawaihae	0.834	0.905	+ 8.5
Wahiawa	0.159	0.154	- 3
Lahaina	0.328	0.308	- 6
Molokai	0.051	0.040	-22
Mahukona	0.116	0.083	-28

Figure 1.

Urease activity in air-dry nonirradiated and irradiated (4 Mrad and 8 Mrad doses) Hawaiian and Puerto Rican latosols; Dublin adobe clay irradiated at 5 Mrad dose (Skujiņš and McLaren, 1969).



Skujins, Figure 1

III. PROGRESS IN RESEARCH

1. FACTORS AFFECTING THE ENZYME ACTIVITY OF EXTRACTED SOIL UREASE

Introduction

Although we have been able to extract a urease-active fraction from Dublin soil,[3] quantitative assessment of the yield has been deferred because the active moiety extracted is apparently under the influence of an inhibitor.[4] It is important to find an effective means of concentrating the active fraction extracted and to remove the inhibitor or at least study its effect upon the enzyme activity. The two methods described previously, namely water extraction with Carbowax and precipitation with calcium chloride, were not satisfactory for reasons mentioned before. A more promising procedure is now described based upon the finding that when a soil extract, obtained by urea-salt combination treatment as described previously, was subjected to extensive dialysis, a sediment possessing considerable urease activity was formed without recourse to precipitation induceable by calcium chloride addition. This sediment does not contain clay (as shown by X-ray diffraction analysis) and has the desirable property of being easy to "clean" (practically all the dark-colored material can be removed without loss of activity). Enzymatic activities of these precipitates appeared to be maintained for a long period of time without deterioration.

The characteristics of these precipitates are studied below from the standpoint of enzyme stability, resistance to various manipulations, and feasibility of desorption and/or dissolution. Any assessment of the amount of activity extracted should be made with these factors in mind. Relative activities are used throughout to facilitate comparison.

Extraction Procedure

A large-scale extraction was carried out in the cold room (≃4°C) as follows. Dublin soil, 3 Kg, was suspended in 10-liter solution having the following composition:

0.25 M Na phosphate buffer at pH 7.0

2 M urea

4 M sodium chloride

and containing

50 g EDTA

50 ml mercaptoethanol

10 ml chloroform

50 ml toluene

The suspension was stirred continuously for 4.5 hours and then filtered through filter candles; 8.5 liters of filtrate was collected (I). To the remaining materials, 10 liters of 0.25 M Na phosphate buffer (pH 7.0) was added, with stirring for 3 hours; a second filtrate was obtained (9.6 liters (II)). Ten liters of 0.05 M Na phosphate buffer (pH 7.0) was subsequently added to the remainder; after stirring for 3 hours 10.4 liters

of filtrate (III) was collected from the candles. An additional filtrate (IV) was obtained by adding 25 liters of 0.05 M Na phosphate buffer (pH 7.0) to the remainder and stirring for 4 hours.

Concentrating the Filtrates

Extensive preliminary investigation with the following columns proved unsatisfactory for practical concentration of the filtrates obtained above.

a. Celite (Johns-Manville)
   a diatomite filter aid

b. AG 1-X_2 (Bio-Rad)
   a quaternary ammonium anion exchange resin

c. Micro-Cell (Johns-Manville)
   a synthetic calcium silicate

d. Cellex-D (Bio-Rad)
   an anion-exchange cellulose

e. Hydroxylapatite (Bio-Rad)
   Bio-Gel.

f. DEAE cellulose in the phosphate form at pH 8.0 appeared to be the best but the results were still impractical.

In all these treatments several experimental conditions were tried with various buffers, with different strengths and pH values. It is not worthwhile to describe these techniques since they proved futile. The following dialysis procedure was most effective in preparing an insoluble fraction from the filtrates.

Sediments, precipitating naturally upon extensive dialysis, were found to exhibit high urease activity. The method adopted as a practical means of concentrating the filtrates is as follows. Two liters of each of the above described filtrates were dialized for 4 days in cellophane tubing under running tap water (in the cold room). Precipitates appeared in all but the first filtrate (I) with the highest amount of sediment in the fourth (IV). These precipitates were collected separately by centrifugation and brought to 10-ml volumes in 0.001 M Na phosphate buffer (pH 7.0). Some characteristics of the filtrates are given in Table 1.

A question arose as to whether the same yield obtains when the three filtrates capable of forming precipitates (Table 1) are combined before dialysis. To this end 3 equal aliquots (1/3 liter each) of these filtrates were mixed in one dialysis bag. One additional aliquot (1/3 liter) from filtrate IV was placed in another bag and the two bags were dialized separately and concentrated as before, keeping the same proportions. The results were surprising in that activity in the combined sample (II + III + IV) amounted only to the activity in sample IV alone. Therefore, combining these filtrates was deemed undesirable for our purpose and it was decided to use filtrate IV exclusively throughout this investigation. The corresponding sediment (IV) will always refer to the material obtained when one liter of the filtrate is brought to 5 ml by the process described above.

Table I

Some properties of precipitates from the successive soil extracts

Filtrate number	Color	pH after dialysis	Relative activity in the precipitate formed*
I	light yellow	8.3	--
II	straw yellow	8.9	1
III	brown	8.7	4
IV	dark brown	9.2	5

*Urease activity determined in 0.25 M Na phosphate buffer at pH 7.0 in Conway dishes as described elsewhere.[3]

Titration of Precipitates

Titration curves depicted in Figures 1 and 2 were obtained with the combined (II + III + IV) sample as follows: 4.5 ml of the concentrate was diluted to 105 ml and brought to pH 3.0 with HCl. The volume was divided into 3 equal parts:

a. Silicates were removed from the first part by Jackson's Procedure[5] as follows. The sample was placed in a centrifuge tube coated with paraffin and centrifuged. The sediment was then washed with 10 ml of 0.12 N HF and made up to the original volume with water and centrifuged. The treatment was repeated 3 times and then the sediment was resuspended in 35 ml of HCl (pH 3.0) and kept to be titrated later.

b. Organic matter was removed from the second part by decomposition with H_2O_2 on a hot plate until the effervescence ceased.

c. The third part was left untreated and titrated directly.

Titration curves of these samples were obtained under nitrogen flow in a Radiometer titrator with the appropriate strength of sodium hydroxide.

Testing for Clay in the Sediment

Samples of concentrate IV were diluted (1/6) and then dried in evaporation dishes in an oven maintained at 80°C. X-ray diffraction was carried out on the dried films by Barshad's method.[1] As pointed out above, no clay was found in these concentrates.

Figure 1. Titration Curves of a combined Sample (II + III + IV) of the Precipitates.

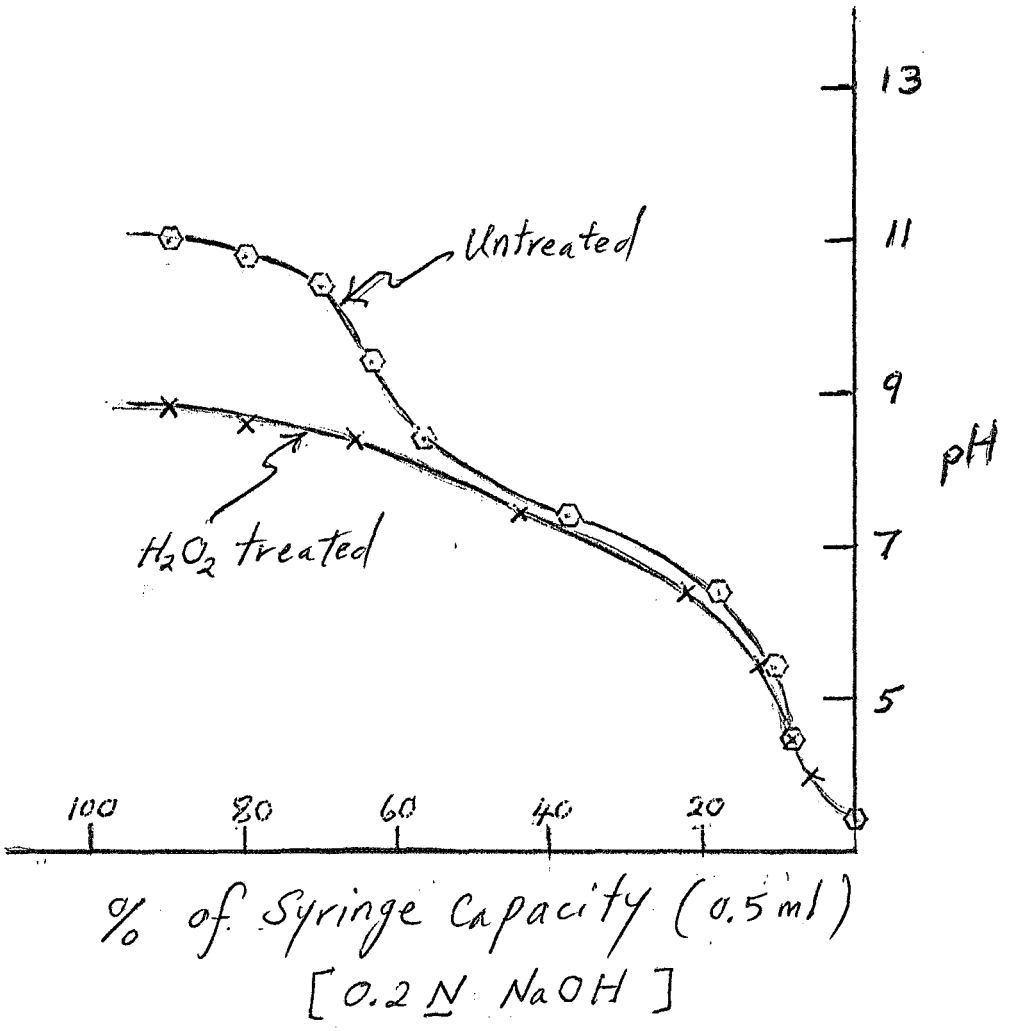
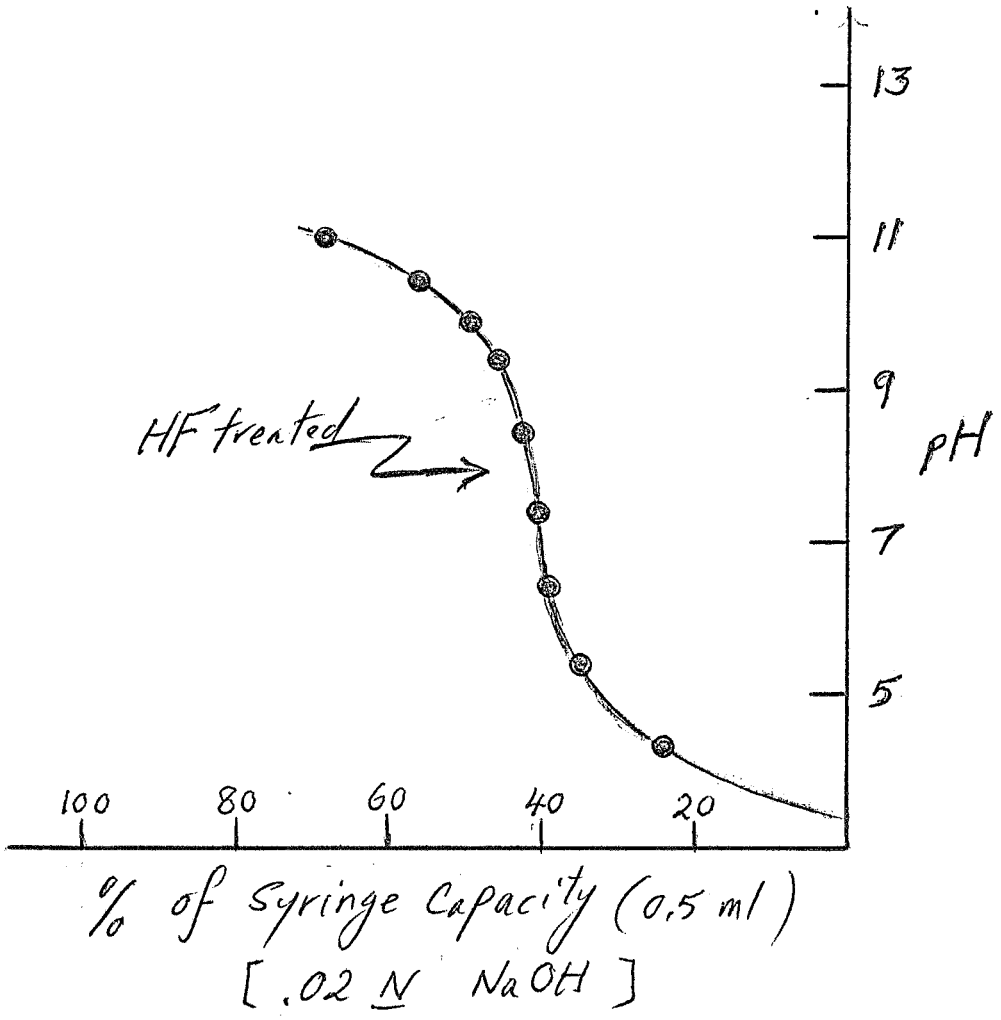


Figure 2. Titration Curve of the Combined Precipitate from which silicates were removed.



Characteristics of Filtrate IV

The following information was obtained through extensive experimentation. We refrain from recording each and every experiment to avoid clumsy repetition.

1. A sample withdrawn from the filtrate (stored at 5°C) two months later and then concentrated exhibited 70% of the original activity.

2. By contrast, the concentrate maintains its activity without detectable loss.

3. Subsequent dialysis of the concentrate against distilled water for one day (after 4 days under running tap water) was accompanied by an increase of about 50% in urease activity, possibly due to the concomitent removal of a salt.

4. Addition of L-cysteine (NBC) to the filtrate before dialysis (in an amount of 10 mg/liter) increased activity of the concentrate by a factor of 3. The "protective" action of this compound cannot be overemphasized.

As an aside, the effect of L-cysteine and β-mercaptoethanol (Eastman Kodak Co.) on soil urease is shown in Figure 3. These results were obtained with 0.2 g soil samples suspended in 4 ml buffer and treated with increasing concentrations of reagent for a period of 10 minutes before an activity measurement. The corresponding effects on Jack Bean urease (Freehold, N.J.) are shown in Figure 4 as obtained with 50 mg samples.

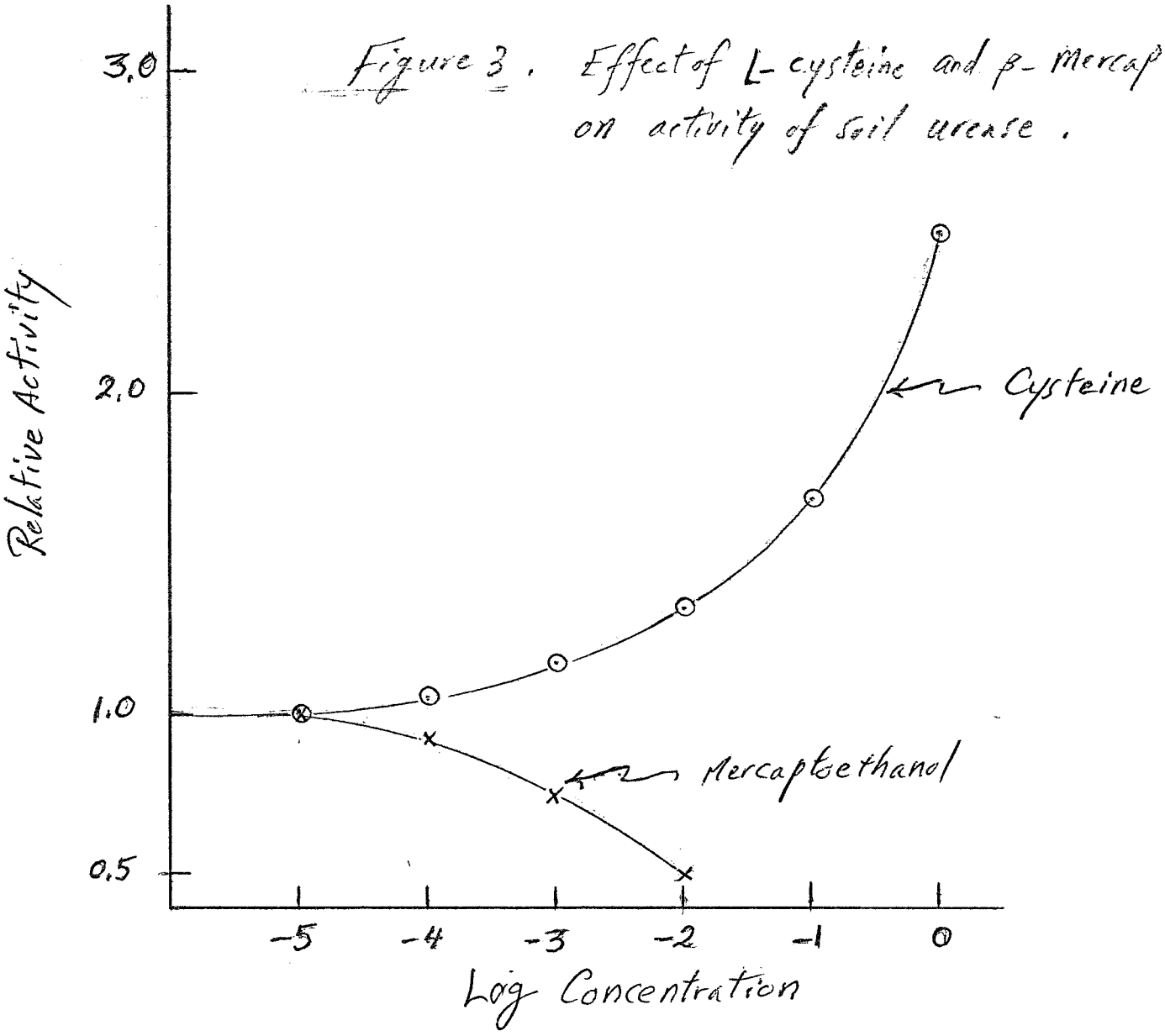
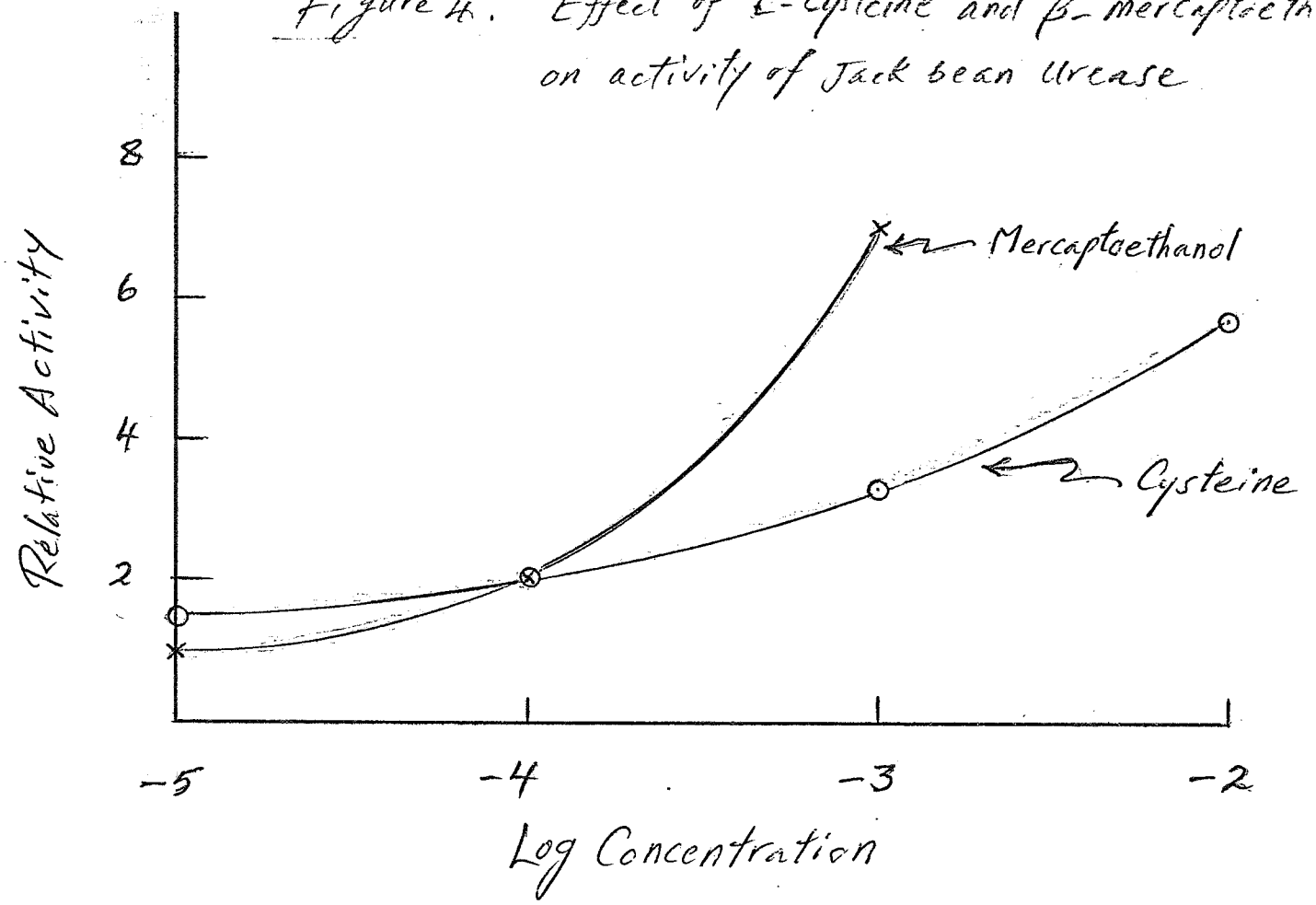


Figure 3. Effect of L-cysteine and β-mercaptoethanol on activity of soil urease.
3.0
2.0
1.0
0.5
Relative Activity
Cysteine
Mercaptoethanol
-5
-4
-3
-2
-1
0
Log Concentration

Figure 4. Effect of L-cysteine and β-mercaptoethanol on activity of Jack bean urease



JBU

Characteristics of Sediment from Filtrate IV

1. Treatment of the sediment (containing cysteine) with 0.25 M Na citrate-phosphate (pH 6.5) resulted in partial solubilization of the sediment. However, the residual sediment (after centrifugation) contained almost all of the original activity; there was practically no activity in the supernates. Upon dializing this sediment against 0.1 M Na phosphate buffer (pH 7.0) overnight, its activity was raised by 60%.

2. Treatment of the sediment with buffer (citrate-phosphate) at 0.8 M concentration resulted in a greater dissolution; the remaining sediment contained 80% of the initial activity. Again, hardly any activity was detected in the supernatant solution (both the sediment and the supernatant were dialized against 0.1 M Na phosphate buffer, pH 7.0, overnight before activity measurements.

3. A treatment similar to that in 2. above, except for the use of a 0.8 M Na phosphate buffer, pH 6.5, (instead of citrate-phosphate) resulted in less dissolution of the precipitate with the sediment containing 90% of the initial activity.

4. With either buffer, the sediment was lighter in color than prior to the treatment.

Effect of Dialysis

Since dialysis of the concentrate was found to increase its activity, it seemed desirable to study the process in more detail.

Time course of dialysis

To 2.5 ml of the concentrate sediment (IV), 7.5 ml of 1 M Na phosphate buffer (pH 6.5) was added; after thorough mixing the suspension was centrifuged. The sediment was made up to 25 ml with 0.1 M Na phosphate buffer (pH 7.0) and divided equally into 5 parts. Four of these were placed in dializing bags to be dialized in beakers, each containing 150 ml distilled water. Dialysis was carried out in a refrigerator for one hour, at the end of which one beaker was removed and dialysates of the remaining beakers were replaced by fresh distilled water (150 ml). Following another hour of dialysis the procedure was repeated with removal of another beaker and so on until the last beaker was removed after 4 hours. At the end of dialysis the suspensions were made up to 12 ml each with 0.1 M Na phosphate buffer (pH 7.0) and their activity obtained (Table 2). It appears that long exposure to distilled water is somewhat detrimental.

Identity of the inhibitor

In order to ascertain whether the inhibitor is either a cation or an anion, use was made of an electrodializer (Alexander and Johnson, Colloid Science, Oxford) provided with VisKing membranes (VisKing Corporation, Chicago 38, Ill.) as follows. To one ml of the concentrate, 3 ml of 1 M Na phosphate buffer (pH 6.5) was added and well mixed for 10 minutes; the suspension was then centrifuged. Five ml of 0.1 M Na phosphate buffer (pH 7.0) was added to the sediment and the resulting suspensions were dialized overnight in 300 ml distilled water and then centrifuged. The

Table 2

Effect of dialysis with replacement in distilled water (after running tap water) on urease activity of the concentrate.

Treatment	Relative activity
No dialysis	1.00
1 hour, 150 ml	1.30
2 hours, 300 ml	1.25
3 hours, 450 ml	1.20
4 hours, 600 ml	1.19

sediment was placed in the central compartment of the electrodializer (300 ml) and dialysed at 100 ma for one hour (the central compartment was stirred meanwhile). The contents of the side compartments (500 ml in each) were than collected separately and replaced by fresh water and dialysis proceeded for an additional 80 minutes. Thus, one liter dialysate was obtained from each side compartment. Each of these solutions and the residual 300 ml in the center compartment was evaporated in a steam bath to a residual volume of 10 m each. The sediment of the central compartment was collected at the end of the electrodialysis and centrifuged; the concentrated sediment was divided into 4 equal parts. Three parts were treated separately with the evaporates obtained above, while the fourth part received 10 ml of distilled water. Urease activity of these samples was then measured by the standard procedure (Table 3).

Although the results are not quite impressive, the indication is that the inhibitory substance is positively charged and of a low molecular size dimension. It remains to be seen whether these cations are either organic or metallic. The inhibitory effect of heavy metal cations on urease has been noted.[6]

Enzyme Desorption from the Sediment

The feasibility of desorbing urease from the sediment by a buffer at a particular pH range was tested as follows. A series of experiments was conducted in which the sediment (IV) was treated with 1 M Na phosphate buffer at various pH values. Although 58% of the activity was lost at pH 5.0, this loss did not show in the supernate. Moreover, prolonged

Table 3

Effect of electrodialysates on urease activity.

Treatment	Relative activity
Control*	1.00
Cathode chamber†	0.76
Middle chamber†	1.13
Anode chamber†	0.85

*Sample not electrodialized.

†Refers to dialysates of the respective chamber.

keeping of the sediment at pH 6.0 proved detrimental to enzyme activity. At pH 8.0 some activity was found in the supernate but the value did not exceed 5% of the initial activity.

Enzyme Release by Dissolution of Precipitate

Attempts to release the enzyme by titrating the sediments with HCl down to pH 5.0 gave very little activity in the supernates. Likewise, adding HF to these precipitates to pH 4 did not prove any better. It was then deemed necessary to first "clean" these materials in the hope of facilitating desorption.

Cleaning the Concentrate

From the foregoing observation, Na phosphate buffer was considered preferable to a Na citrate-phosphate combination and the following experiment was performed exclusively with this particular buffer at a strength of 1 M and pH 6.5. Thirty ml of buffer was added to 5 ml of the concentrate (sediment IV) and, after mixing well for 10 minutes, the suspension was divided equally into 7 centrifuge tubes. These tubes were treated alternately as indicated in Table 4, with buffer and centrifugation.

Thus, we are left with a yellowish-gray sediment retaining nearly all the original activity in spite of removing the "dirty" material. This is a fortunate accomplishment since these colored materials constituted an obstacle during previous attempts to concentrate the extracts.

Table 4

Urease activity of the concentrate as a function of successive washing with sodium phosphate buffer.

Treatment (washing)*	Color of supernate	Relative activity of remaining sediment
None	--	1.00
Once with 1 M	very dark brown	1.23
Twice with 1 M	brown	1.23
(X) Thrice with 1 M	light brown	1.19
(X) + once with 0.25 M	almost colorless	1.02
(X) + twice with 0.25 M	almost colorless	1.02
(X) + thrice with 0.25 M	almost colorless	1.02

*5-ml aliquots were used in each washing.

Extraction of Urease from the Cleaned Sediment

Two-ml aliquots of the original sediments were placed in 4 centrifuge tubes. To each tube was added a 4-ml aliquot of 1 M Na phosphate buffer at pH 6.5 and, after mixing for 10 minutes, the tubes were centrifuged. The supernatants were discarded. The process was repeated 4 times following which one of the following reagents was added:

Tube a. 1 M Na phosphate pH 5.0, 4 ml

Tube b. 1 M Na phosphate pH 8.0, 4 ml

Tube c. 40% ammonium sulfate solution, 4 ml

The tubes were shaken and centrifuged and the supernatants were collected and dialized against 0.1 M Na phosphate buffer (pH 7.0) overnight in a cold room. Urease activity in these supernates was measured. When it was realized that the phosphate buffer at pH 8.0 was the best extractant (second column in Table 5), the remaining sediments were further extracted with this solution (third column in Table 5). Activity in the remaining sediments was determined by standard procedure. Because of the observed increase in their activity (Table 6), the actual percentages of extraction were recalculated.

It may not be appropriate to make the following quantitative assessment of the amount of enzyme extracted from the soil by the method described since the yield has yet to be maximized. But at this stage, and solely on the basis of enzyme activity, the following may be arbitrarily stated. When cysteine was added, urease activity in the 25 liters of filtrate IV amounts to 11% of the original activity in soil.

Table 5

The relative effectiveness of various extractants to release the enzyme from the sediment.

Treatment*	Activity in the extract**	
	First	Second
a	1.8	11
b	17.5	34
c	3.4	20

*See text.

**Percentage of initial activity in the sediment.

Table 6

Total enzyme released after the two successive extractions reported in Table 5.

Treatment	% increase in activity of the sediment*	% extracted**
a	243	5.0
b	227	18.4
c	200	10.5

* Over the initial activity of the sediment before treatment.

**Sum of the respective two values in Table 5 corrected for the concomitent increase of sediment's activity tabulated above.

Using the proportions in Table 1 and assuming that the other filtrates behave similarly (they have yet to be investigated by this method) the total percentage extracted should be double that figure.

Urease Activity and Microbial Counts

There exists a definite quantity of native urease in the soil in addition to that pertaining to microbial growth. Figure 5 shows this clearly and supplements data in a preceding report.[4] The previous data pertain to total numbers of microorganisms and not to ureolytic species as was mistakenly written. The total number of microorganisms was evaluated by the dilution-plate method[2] with soil extract agar. The number of ureolytic organisms was obtained using urea as the sole source of carbon and nitrogen by a procedure given by Paulson and Kurtz.[7]

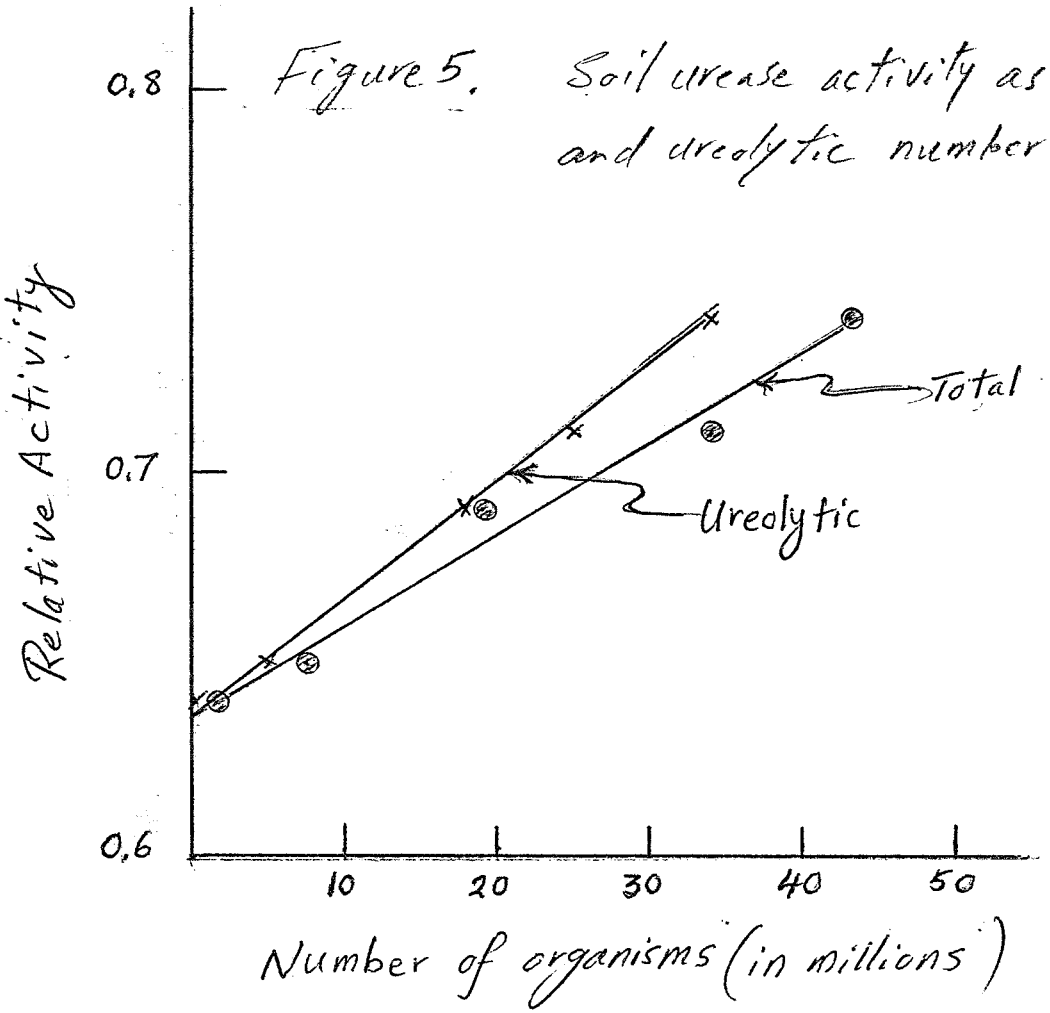


Figure 5. Soil urease activity as a function of total and ureolytic number of microorganisms

SUMMARY OF UREASE EXTRACTION FROM DUBLIN SOIL BY THE UREA-SALT COMBINATION

1. A urease-active organic fraction can be detached from Dublin soil by a solution of 2 M urea and 4 M NaCl buffered with either Tris or phosphate at pH 7.0.

2. Subsequent extraction of the once-treated soil with buffer alone brings the detached organic matter into solution in successive stages, especially when a more dilute buffer is used.

3. Clay can be removed from these fractions by filtration of the extracts through candles and the urea and salt can be removed by dialysis.

4. Addition of cysteine to the filtrates enhances enzymatic activity.

5. Extensive dialysis against running tap water results in precipitation of a dark sediment possessing high urease activity.

6. From this sediment almost all the dark material can be removed through repeated washing with a solution of 1 M Na citrate-phosphate buffered at pH 6.5.

7. A portion of the enzyme in this cleaned sediment can be released with either 1 M Na phosphate solution at pH 8.0 or with a 40% ammonium sulfate solution.

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2. Concerning the Origin, Location and Persistence of Soil Urease

Introduction

Most of the basic problems concerning the origin, location and persistence of soil enzymes remain unsolved. For example, enzymes in soil are generally more resistent to breakdown by other proteinases than those in vitro. This persistence of enzymes has been attributed to either internal and external adsorption onto clay colloids (Ensminger and Gieseking 1942; Pinck, Dyal and Allison 1954) or to the formation of resistent enzyme-organic matter complexes (Conrad 1940; McLaren 1963). Specifically, the high affinity between colloids and urease has lead to the suggestion that adsorption can protect this enzyme from breakdown in soil (Durand 1964 and 1965).

Addition of urease to soil increases urea hydrolysis only temporarily (Conrad 1940; Moe 1967; Stojanovic 1959; Roberge 1970). This suggests that either the added urease is inactivated by proteolysis or by adsorption. In either case the presence of a constant background level of enzyme activity in soil, independent of microbial proliferation, indicates that some protective yet uninhibiting mechanism is extant (Paulson and Kurtz 1969). In fact, urease activity has been detected in soils stored for decades, and correlates better with organic matter contents than with numbers of microbes (Skujins and McLaren 1969).

Herein we illucidate the location of some of the urease activity in a soil and suggest a reason for its persistence along the lines indicated by the pioneer effort of Conrad.

Materials and Methods

i Soil

A Dublin loam soil with the following characteristics was used: sand 24%, silt 35%, clay 41%, organic matter 2.9% and a pH of 7.2. In experiments with soil suspensions the sand fraction was allowed to sediment out before use. Consequently 1 ml of the residual suspension contained 0.035 g silt, 0.041 g clay and 0.00292 g organic matter.

ii Measurement of Enzyme Activity

Soil urease, commercial urease, and pronase (from Streptomyces griseus) activities were determined by ammonia production using urea and benzoyl arginine amide respectively as substrates, in a modified Conway diffusion dish (Obrink 1955), as detailed by McLaren, Reshetko and Huber (1957). The 3XN.F urease was purchased from Nutritional Biochemical Corporation and the B-grade pronase was purchased from Calbiochem. The latter is known to contain several enzymes (Nomoto, Narahasi and Murakami 1960).

iii Organic Matter Extraction

Urea - Although concentrated urea is a denaturating agent for enzymes, it can be used to extract organic matter from soil (Barker, Hayes, Simmonds and Stacy 1967). Since enzyme denaturation by urea is

supressed by high salt concentration (Malhorta and Rani 1969), the following extraction procedure was developed (El Sayed and McLaren 1970). Soil, 200 g, is suspended in one liter of aqueous urea (2M) and sodium chloride (4M) buffered at pH 7.0 with 0.25M sodium phosphate and 1 g of EDTA. Mercaptoethanol, 1 ml, is added since soil ureases may be SH-enzymes (Skujins and McLaren 1968). The suspension is shaken under refrigeration for four hours and then centrifuged to collect the first extract. Sedimented material is extracted a second time with a liter of 0.25M sodium phosphate buffer at pH 7.0, a third time with a liter of 0.05M sodium phosphate, and finally with two liters of buffer. The extracts, successively darker in colour, are each subjected to dialysis under running water in a refrigerator for 96 hours.

Sonication - Methods of separating soil fractions by sonication have been used frequently in recent years (Felbeck 1959; Halstead, Anderson and Scott 1966; Edwards and Bremner 1967; Burns and Audus 1970). Hence an attempt was made to separate enzyme-active organic material from soil following treatment with a Circo 60 watt ultrasonic generator. Dublin soil, 50 g, suspended in 500 ml of water, was subjected to four hours sonication. The resulting suspension was centrifuged at 14,000 g and the golden-brown supernatant sol was tested for urease activity.

iv Preparation of a Bentonite-Urease Complex (BUC)

One ml of urease at pH 7.0 was added to 0.1 g of bentonite clay (0.005 or 0.01 g of enzyme per 1 g clay) in the outer diffusion chamber of a Conway dish. After six hours, maximum expansion of the lattices was

considered to be complete (Estermann, Peterson and McLaren 1959) and the urease activity of BUC was tested as described above (ii). The stability of BUC toward proteolysis was also tested following the addition of 1 ml of a 500 ppm pronase solution with an allowance of twelve hours for reaction.

v Preparation of a Bentonite-Urease-Lignin Complex (BULC)

Urease, 10 ml, was added to 5 g of bentonite (0.001 g of enzyme per 1 g clay). After allowing six hours for adsorption to take place, 10 ml of lignin (Estermann, et al. 1959) was added (0.01 g lignin per 1 g clay) and the resulting complex was mixed thoroughly into a paste. This paste was allowed to dry at room temperature for 72 hours and then ground to a fine powder. The procedure as used in ii was followed for estimating urease activity. Preparations of bentonite-urease, bentonite-lignin and bentonite-water were used as controls.

Results

i Action of Pronase on Commercial Urease Activity

Table 1 shows the loss in urease activity caused by the addition of an equal concentration (500 ppm) of pronase. The components were allowed to react in pH 7.0 buffer for 20 hours in a constant temperature water bath at 37°C. The results are corrected for controls and indicate that the proteolytic enzyme pronase is very active in inactivating urease.

ii The Effect of Pronase on Soil Urease Activity

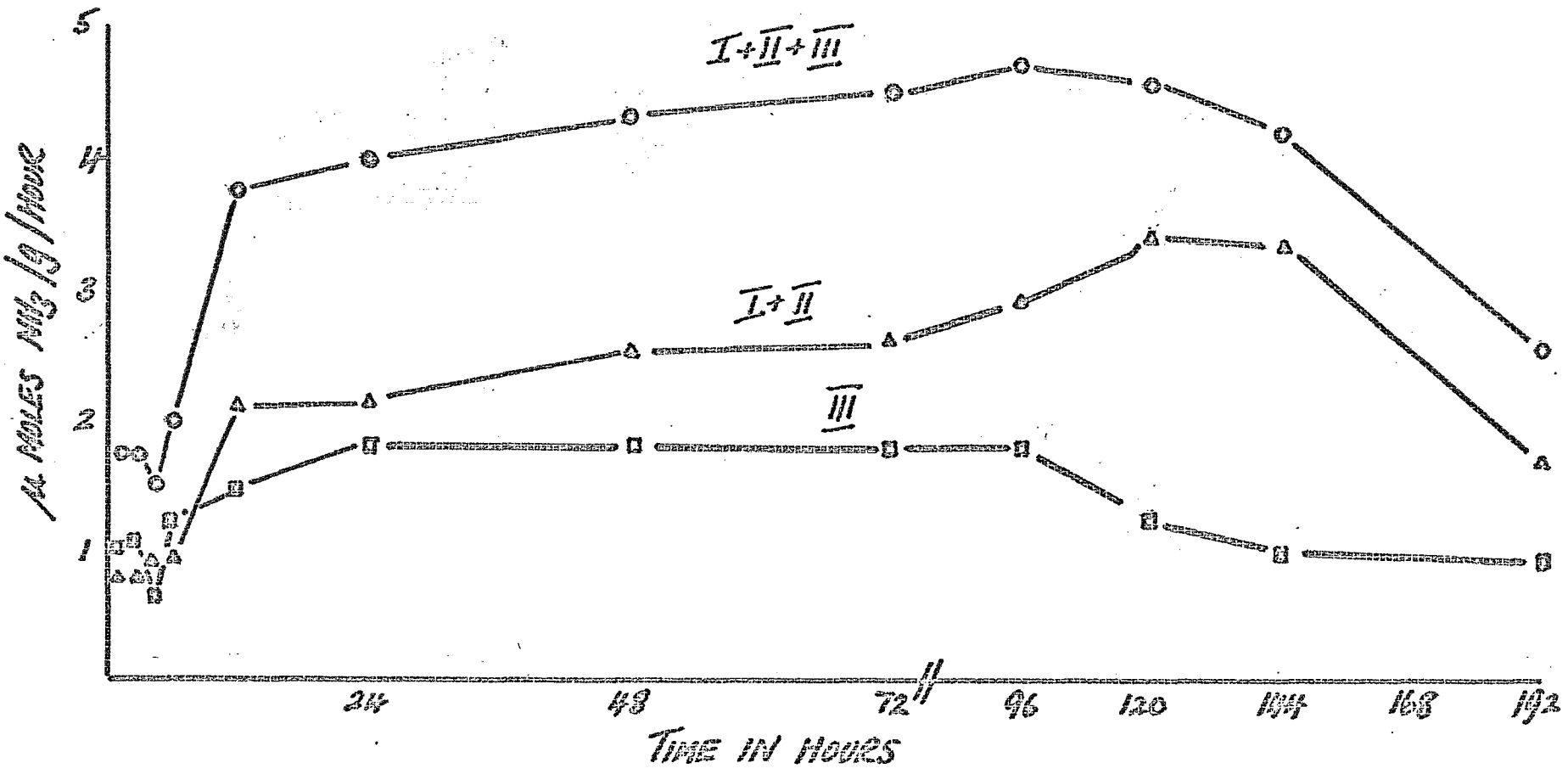
In order to discover if very high proportions of proteolytic enzyme would inactivate soil urease, when incubated at 37°C for 24 hours, two weight ratios of pronase to organic matter were used: 3.3:1 and 0.7:1 (Ladd and Brisbane, 1967, used 1:2.4-1:24 in their experiments). The results, in Table 2, show that at both pronase concentrations soil urease activity is unimpeded.

Soil and pronase (1 pronase : 30 organic matter) were allowed to react at 37°C for 24 and 336 hours, after which times urease activity was measured. Clearly urease activity in soil is resistent to pronase with any time of exposure, since results predicted on the basis of no activity were observed (Table 3).

iii Persistence of Pronase in Soil

In order to measure the persistence of pronase added to soil 5 ml of soil suspension (0.3946 g soil) and 1 ml of pronase (0.0001 g enzyme) were incubated together at 37°C. 0.3M benzoylargenine amide (BAA) was added to 1 ml samples and the subsequent release of ammonia used as a measure of pronase activity. The results are represented graphically in Fig. 1. The total ammonia released appears to consist of three portions: that released by the breakdown of pronase, probably by soil enzymes (I); that from the effect of pronase on nitrogen-containing substrates already present in soil (II), and ammonia produced as pronase reacts with BAA added to soil (III). The first two are inseperable under the conditions of this experiment and are grouped together as

Fig. 1. THE PERSISTENCE OF PRONASE IN SOIL



ammonia from pronase. The rate of ammonia production by pronase (I and II) exhibits a distinct lag period during the first six hours of incubation. This lag may represent ammonia production by microbial enzyme breakdown of pronase, but note evidence of a short lag in ammonia production in the pronase-BAA component (III), which is contrary to this explanation. In any case, the data in this figure reveal that pronase activity is nearly constant from between 1 and 4 days, i.e., it is active and present and could act on soil urease if the urease were not protected (cf. i above).

iv Activity of Urease Added to Soil

Length of pronase treatment - A 10 ml suspension of soil (0.7892 g) and 10 ml urease (500 ppm buffered at pH 7.0) was allowed to incubate for 24 hours. The reduction in activity due to subsequent exposure to 500 ppm pronase is shown in Figure 2. It can be seen that additional urease applied to soil was not protected from pronase attack. The base level of soil urease activity was unaffected by pronase.

Length of adsorption time - Soil suspensions and urease (see iv) were mixed and allowed to react for times ranging from 0 to 120 hours and then subjected to a six hour pronase treatment (500 ppm). Figure 3 shows that a large additional reduction in urease activity is caused by pronase regardless of the pre-incubation time and that the basic soil urease level was unaffected. The soil plus urease sans pronase treatment, a control, shows an initial high activity but indigenous proteolytic enzyme activity soon reduces the amount of activity to that of the untreated soil.

Fig. 2 THE PERSISTENCE OF UREASE ADDED TO SOIL RELATED TO LENGTH OF PRONASE TREATMENT

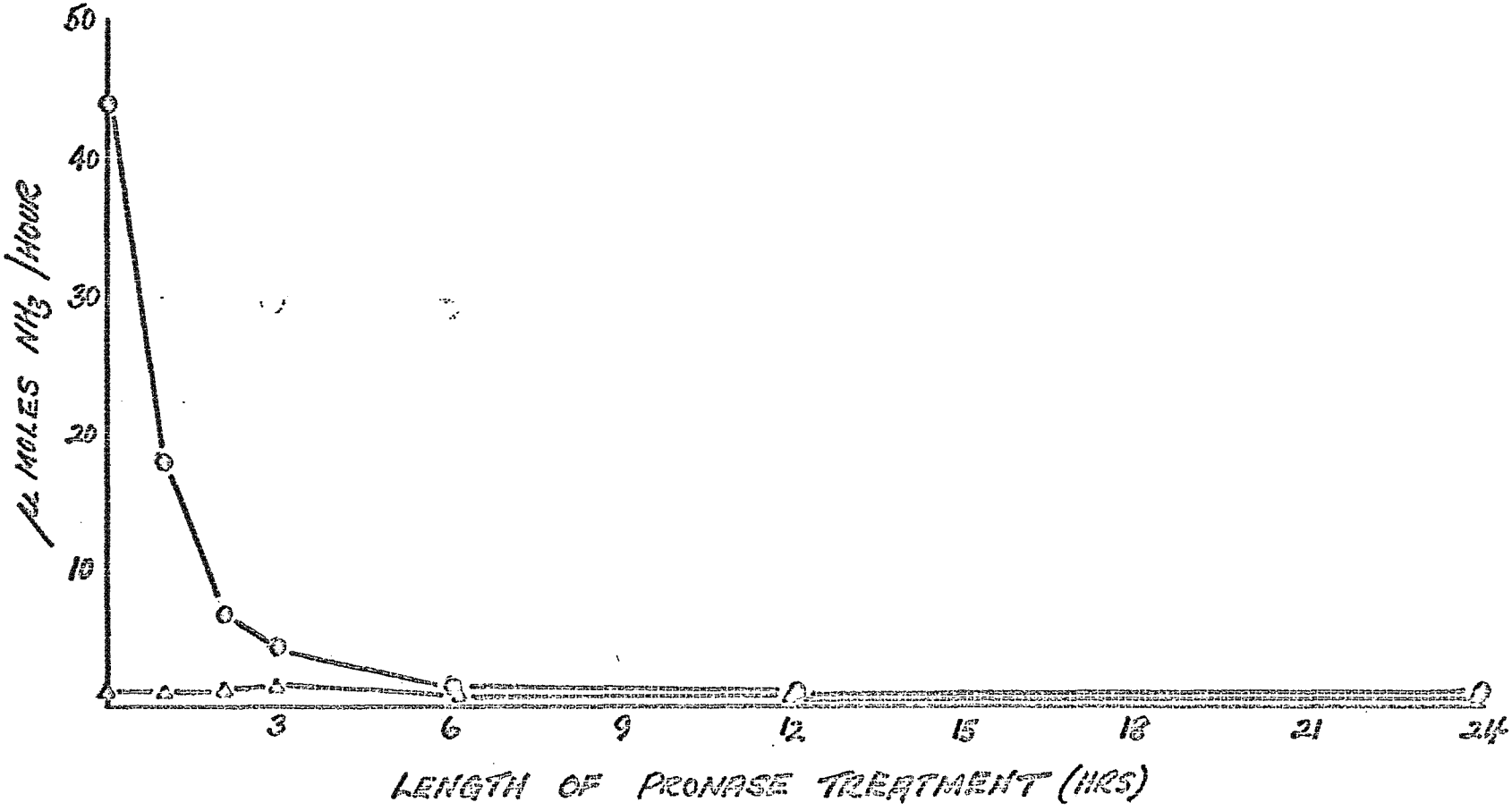
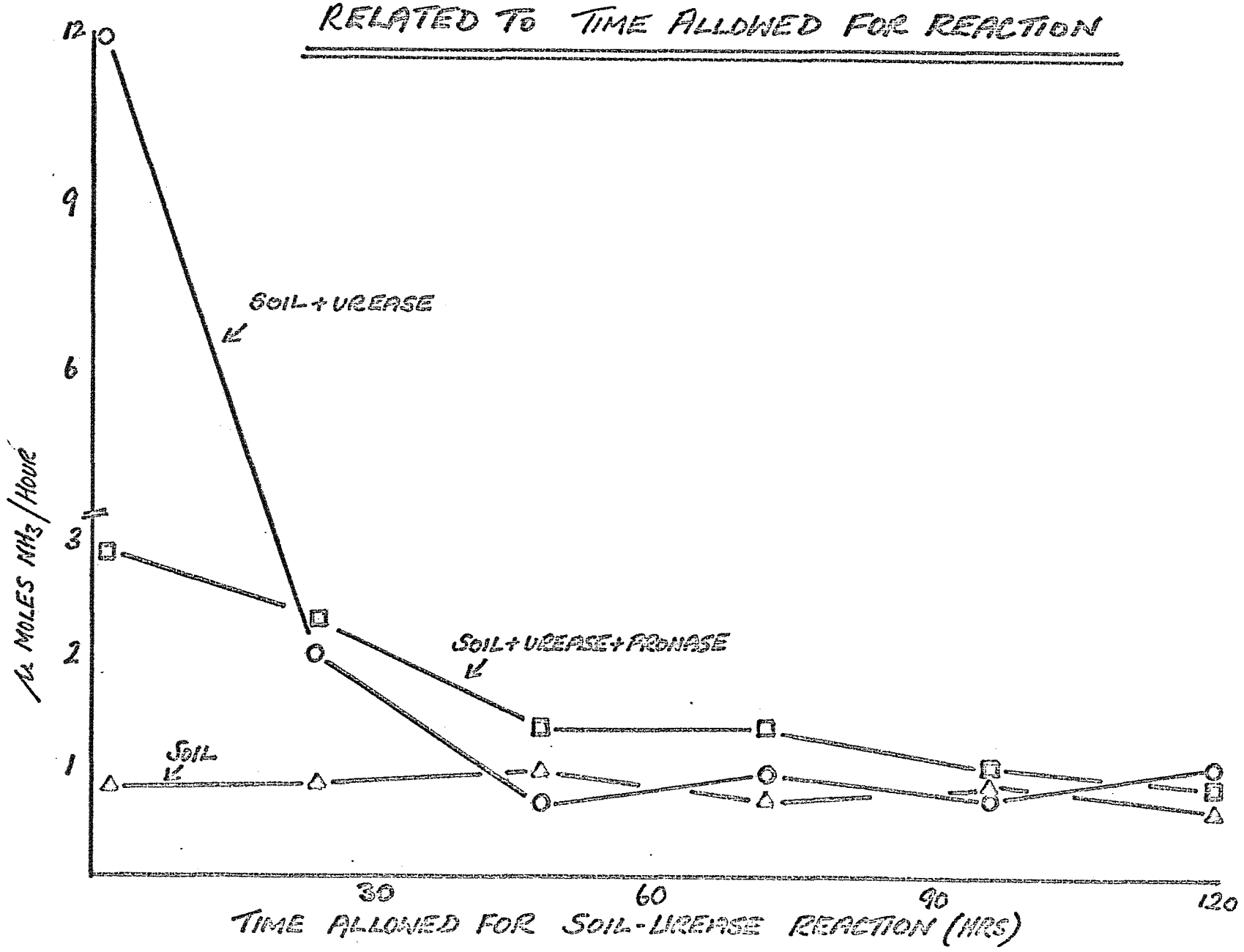
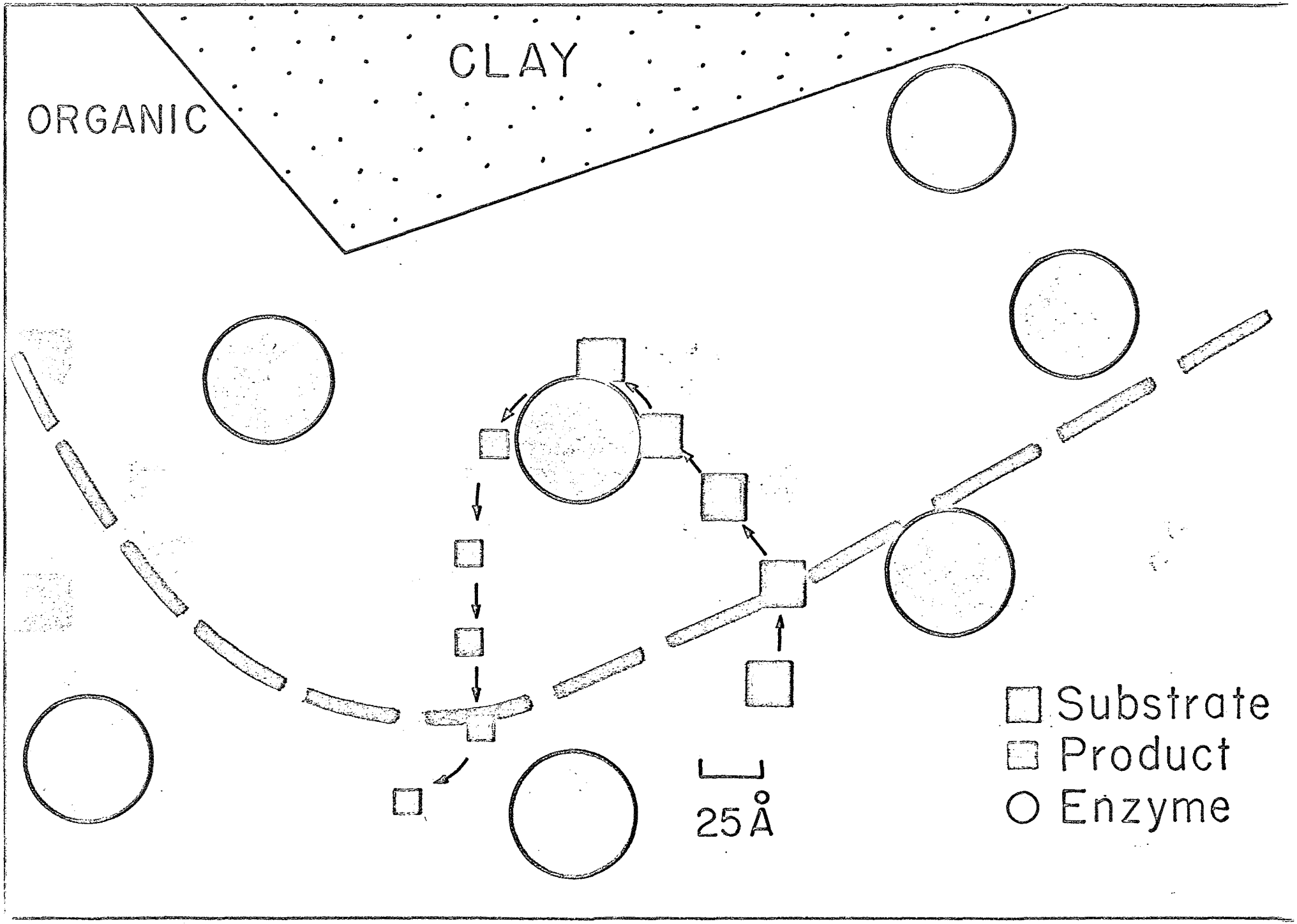


Fig. 3 THE PERSISTENCE OF UREASE ADDED TO SOIL RELATED TO TIME ALLOWED FOR REACTION




CLAY
ORGANIC
Substrate
Product
Enzyme
25 Å

v Activity of Extracted Organic Matter

Organic matter extracted by both the urea method and by sonication showed enzyme activity. By X-ray analyses it was found that the urea extract was devoid of clays whereas the sonicated sample had a high clay content. Although urease activity was higher in the urea extract than in the sonicated extract, urease activity in both was resistant to attack by pronase (Table 4).

vi Stability of the Bentonite-Urease Complex (BUC) in the presence of Pronase

A twelve hour exposure of BUC to pronase greatly reduced urease activity, Table 5. Note that BUC actually exhibited greater activity than urease alone.

vii Stability of the Bentonite-Urease-Lignin Complex (BULC)

Table 6 shows an overall reduction in urease activity following addition of lignin to the BUC but no further reduction occurred on exposure to pronase. The controls (BUC), cf. i, exhibited a marked reduction in urease activity upon addition of pronase.

Discussion

The proteolytic enzyme mixture (from *Streptomyces griseus*) "pronase" is very active in the breakdown of Jackbean urease and other proteins. We would therefore expect pronase to hydrolyse soil proteins, including urease unless some mechanisms for "protection" of soil proteins are extant.

Clearly, in soil pronase does not deactivate urease activity, regardless of the concentration or time of contact. It is therefore evident that urease is shielded from the normal proteolytic effects of pronase. It is also obvious that this protective mechanism does not prevent soil urease - urea interaction.

Pronase added to soil is not stable, but its activity is persistent enough to attack soil urease if the enzymes could combine. On the other hand, urease added to soil is not resistant to proteolytic attack by either soil enzymes or added pronase and therefore increases in urea turnover are not prolonged. This lack of resistance is in part due to proteolysis by soil proteases but is enhanced by subjecting the soil to pronase treatment.

The organic matter extracted by the urea method was free of clays and yet showed a urease activity resistant to pronase. This indicates that urease, in this instance, is primarily associated with the soil organic matter and not with the clay colloids. Sonicated extracts also showed a urease activity resistant to pronase and the results obtained by the two methods indicate that clays (present in the sonicated extract) are not required for protection of urease activity from pronase.

In fact, bentonite clay alone does not protect Jackbean urease from pronase. The observation that this urease activity was increased by bentonite is contrary to many reports indicating a reduction in enzyme activity upon adsorption (Durand 1964; Paulson and Kurtz 1970). At this stage it is difficult to explain this observation and, as it is outside the main line of the present investigation, it is suffice to mention

that this urease may dissociate upon adsorption and this could expose many more active sites than present in polymeric form in solution (Reithel and Robbins 1967).

Addition of a lignin to the bentonite-urease complex affords protection to urease from pronase attack. All of our results with soils and models suggest that enzymes exist in soil as enzyme-organic matter complexes. This association protects the enzyme from attack by other enzymes and yet allows diffusion of substrate and product molecules to and from the active enzyme sites. In the soil the colloidal organic matter is associated with minerals and it has been suggested that the enzymes are situated within that organic matter per se (McLaren 1963, 1970). The nature of this association is represented schematically in Fig. 4. Evidence from the non-persistence of urease added to soil, the bentonite-urease mixture, and the persistence of this enzyme in soil and in a bentonite-urease-lignin complex suggests that for an enzyme to be persistent in soil it needs to be incorporated into the organo-mineral complex. Presumably, as the enzymes are liberated during digestion of plant roots, microorganisms, etc., they are incorporated into the soil organic matter undergoing simultaneous synthesis (Konanova 1961). Synthetic high polymer-enzyme systems are well known and have similar properties (McLaren 1970).

This hypothesis goes some way towards explaining the site and persistence of enzyme activity in soil whilst the authors realise that a considerable amount of ephemeral enzyme activity may be due to free, unassociated enzymes in soil (Briggs and Spedding 1963).

Table 1

The activity of pronase and non-pronase treated urease in vitro.

μ moles NH_3 evolved / hour	
Urease + Urea	Urease + Pronase + Urea
54.48	0.64

Table 2

The effect of pronase on soil urease activity

Pronase: Organic Matter	μ moles NH_3 / g / hour				
	No Pronase		Pronase		
	Soil (A)	Soil & Urea (B)	Soil (C)	Soil & Urea (D)	Expected* (D)
3.3:1	0.12	0.25	0.35	0.44	0.47
0.7:1	0.12	0.25	0.16	0.27	0.29

* A + (B - A) + (C - A) = D = D Expected if there is no reduction in urease activity due to addition of pronase.

Table 3

The effect of length of pronase treatment on soil urease activity

Time (hrs)	μ moles NH_3 / g / hour				
	No Pronase		Pronase		
	Soil (A)	Soil & Urea (B)	Soil (C)	Soil & Urea (D)	Expected (D)
24	0.02	0.28	0.34	0.58	0.60
336	0.02	0.16	0.44	0.60	0.58

Table 4

Urease activity and its persistence in soil extracts

Treatment	μ moles NH_3 / hour	
	Sonicated Extract	Urea Extract
Extract/Pronase/Urea	1.46	1.98
Extract/Urea	0.32	1.46
Extract/Pronase	1.14	0.36

Table 5

Stability of the bentonite-urease complex

Urease (g/g of soil)	μ moles NH_3 / hour			
	No Pronase		Pronase	
	Bentonite + Urease	Urease	Bentonite + Urease	Urease
0.005	69.28	12.48	34.88	1.46
0.01	262.04	40.28	70.76	1.56

Table 6

Stability of the bentonite-urease-lignin complex

μ moles NH_3 / hour							
No Pronase				Pronase			
BULC	BUC	BL	B	BULC	BUC	BL	B
1.20	9.04	0.64	0.60	1.60	3.76	1.00	0.86

B = Bentonite
U = Urease
L - Lignin
C = Complex

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3. PHOSPHATASE ACTIVITY OF DUBLIN SOIL

This report continues an investigation of the characteristics of phosphatase activity of Dublin soil. The variation in the phosphatase activity of the soil with substrate concentration and with pH was determined. In addition, columns of soil crumbs were perfused with solutions of substrate and the variation in the rate of of reaction with substrate concentration and with flow rate was determined. The relationship between the rate of the phosphatase reaction and the substrate concentration for the soil and for the column of crumbs is discussed.

Materials and methods

Substrate

Disodium para-nitrophenol phosphate (Calbiochem, Los Angeles) was used as substrate.

Soil columns

The columns of soil crumbs were prepared as before (3) except that the columns were prepared in 0.01 M sodium maleate buffer pH 6.90 and the glass columns which held the crumbs were placed in a jacket through which water at 25.0 ±0.1 °C was circulated during preparation of the column and during perfusion.

Determination of pH

Reaction mixtures were prepared and treated exactly as for the determination of phosphatase activity. After the incubation period the tubes were shaken vigorously,

the contents poured into a small beaker, and the pH of the mixtures was measured immediately on a Beckman Zeromatic pH meter, Model 96.

Other materials and methods as before (3).

Results

A. Phosphatase activity of New Dublin soil at different concentrations of substrate

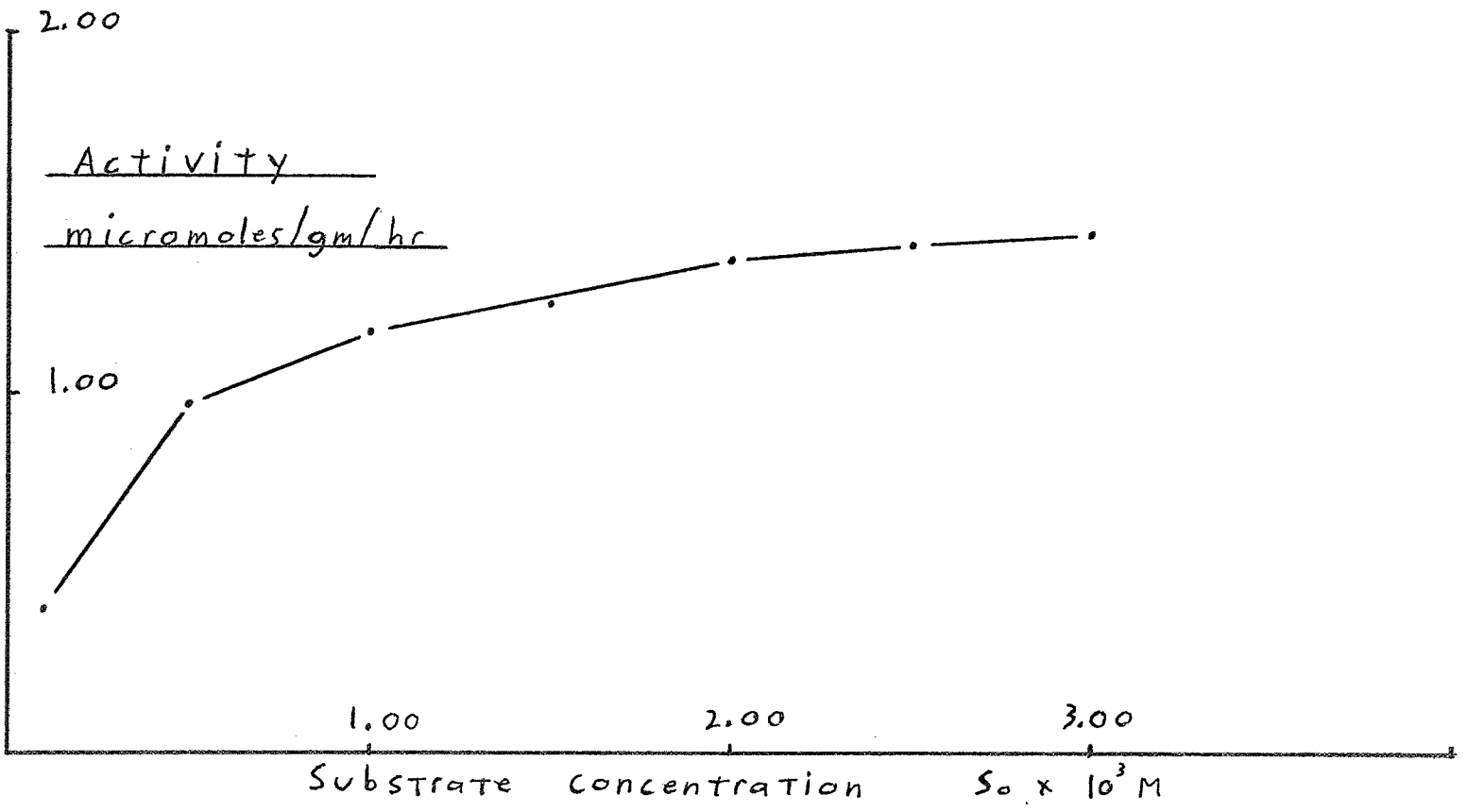
The phosphatase activity of New Dublin soil was determined at various concentrations of substrate. The procedure used was that for the determination of the activity of the soil in suspension (3). The reaction mixtures contained 1.0 gm soil, 0.80 to 24 micromoles of substrate, 2.0 ml of 0.04 M sodium maleate buffer pH 6.88, and distilled water to 8.0 ml, in screw-capped tubes. The pH of the reaction mixtures was 6.90. The reaction was allowed to proceed 1.0 hr at 25.0 °C with end-over-end agitation of the tubes. All tubes were prepared in duplicate and the phosphatase activity was expressed as the average of the duplicate samples. The results of the experiment are shown in Figure 1.

In Figure 1 the phosphatase activity of New Dublin soil is plotted against the substrate concentration in the reaction tubes. The data show that the activity of the soil increased with increasing substrate concentration, appearing to approach a constant value at substrate concentrations somewhat higher than 3.00×10^{-3} M. At a substrate concentration of 3.00×10^{-3} M the phosphatase activity of the soil was 1.44 micromoles/gm/hr.

Under the conditions of the experiment, the phosphatase activity of New Dublin soil became relatively independent of substrate concentration at a concentration of 3.00×10^{-3} M.

FIGURE I

PHOSPHATASE ACTIVITY OF SOIL AT VARIOUS CONCENTRATIONS OF SUBSTRATE



It was decided, therefore, to adopt the conditions used in this experiment, and a substrate concentration of 3.00×10^{-3} M as standards under which the phosphatase activity of New Dublin soil in suspension would be determined.

B. Phosphatase activity of New Dublin soil under standard conditions

The phosphatase activity of New Dublin soil in suspension was determined under the standard conditions described above (section A). Twelve 1.0 gm samples of soil were analyzed in five separate experiments. The results of the experiments are shown in Table 1.

Table 1. Phosphatase activity of New Dublin soil in suspension

Expt.	phosphatase activity umoles/gm/hr	average activity umoles/gm/hr	average deviation from mean umoles/gm/hr
1	1.47 1.43	1.45	0.02
2	1.35 1.39	1.37	0.02
3	1.32 1.41	1.37	0.05
4	1.35 1.41	1.38	0.03
5	1.37 1.43 1.45 1.41	1.41	0.03

The average activity of the twelve samples was 1.40 micromoles/gm/hr and the average deviation was 0.04 micromoles/gm/hr. The average value for activity from two samples of soil appears to provide a sufficiently accurate measure of the activity of the soil, within the experimental variation for the twelve samples.

C. Reaction controls in the determination of phosphatase activity of New Dublin soil in suspension

The activity occuring in two controls for the determination of the phosphatase activity of New Dublin soil was determined. In the first control, the phosphatase activity was measured in samples to which alkali had been added to stop the reaction. In the second control, the phosphatase activity of autoclaved soil was determined. The phosphatase activity was determined using the standard conditions described previously (section A) but with the following modifications:

I. Activity of alkalized samples on standing:

Two sets of tubes were run simultaneously. Each set contained the reaction mixtures and the controls in quadruplicate. After the one-hour incubation period the reaction was stopped in all tubes by adding 2.0 ml of 0.5 M NaOH. One set of tubes was analyzed immediately; the other set was analyzed after standing undisturbed at 25.0 °C for four hours. The phosphatase activity of each set is expressed as the average of the

quadruplicate samples.

II. Activity of autoclaved soil:

Before adding the other reagents, the soil in the tubes was spread the length of the tube and the tubes were autoclaved 3.0 hr at 122 °C. The tubes were prepared in triplicate and the phosphatase activity is expressed as the average of the triplicate samples.

The results of the two experiments are shown in Table 2.

Table 2. Phosphatase activity in reaction controls.

treatment	average phosphatase activity umoles/gm/hr
analyzed immediately	1.41 ± 0.03
analyzed after four hours	1.44 ± 0.04
autoclaved soil	0.010 ± 0.007

In the samples analyzed immediately the average activity was 1.41 micromoles/gm/hr with an average deviation of 0.03 micromoles/gm/hr. In the tubes analyzed after standing four hours the average activity was 1.44 micromoles/gm/hr with an average deviation of 0.04 micromoles/gm/hr. The activity that occurred in the alkalized samples was therefore (1.44 - 1.41)/4 = 0.0075 micromoles/gm/hr.

In the usual determinations of phosphatase activity the samples are analyzed less than an hour after stopping the reaction. The average deviation in the measured values

of activity of New Dublin soil is 0.04 micromoles/gm/hr. Since 0.0075 micromoles/gm/hr is less than 0.04 micromoles/gm/hr, the activity that occurs in the alkalized samples is negligible compared to the variations in values normally encountered for the activity of New Dublin soil, and so no error is introduced into the value for activity by activity occuring in the reaction tubes after the reaction has been stopped.

For the second control, the data indicate that autoclaving the soil almost completely destroyed the phosphatase activity of the soil (Table 2). The activity of the autoclaved soil was 0.010 micromoles/gm/hr, a value which is less than 1% of the activity of the soil not autoclaved. The results indicate that the phosphatase activity of the soil resides in a heat-labile part of the soil organic matter rather than in the inorganic material since the heat treatment would not appreciably alter the properties of this latter fraction.

The phosphatase activity of a sample of a different Dublin soil was also reduced to less than 1% by autoclaving three hours, in a previous experiment (2).

D. Phosphatase activity of New Dublin soil at various pH values.

The phosphatase activity of New Dublin soil at various pH values was determined. The procedure for the determination of the activity of the soil in suspension was followed (3).

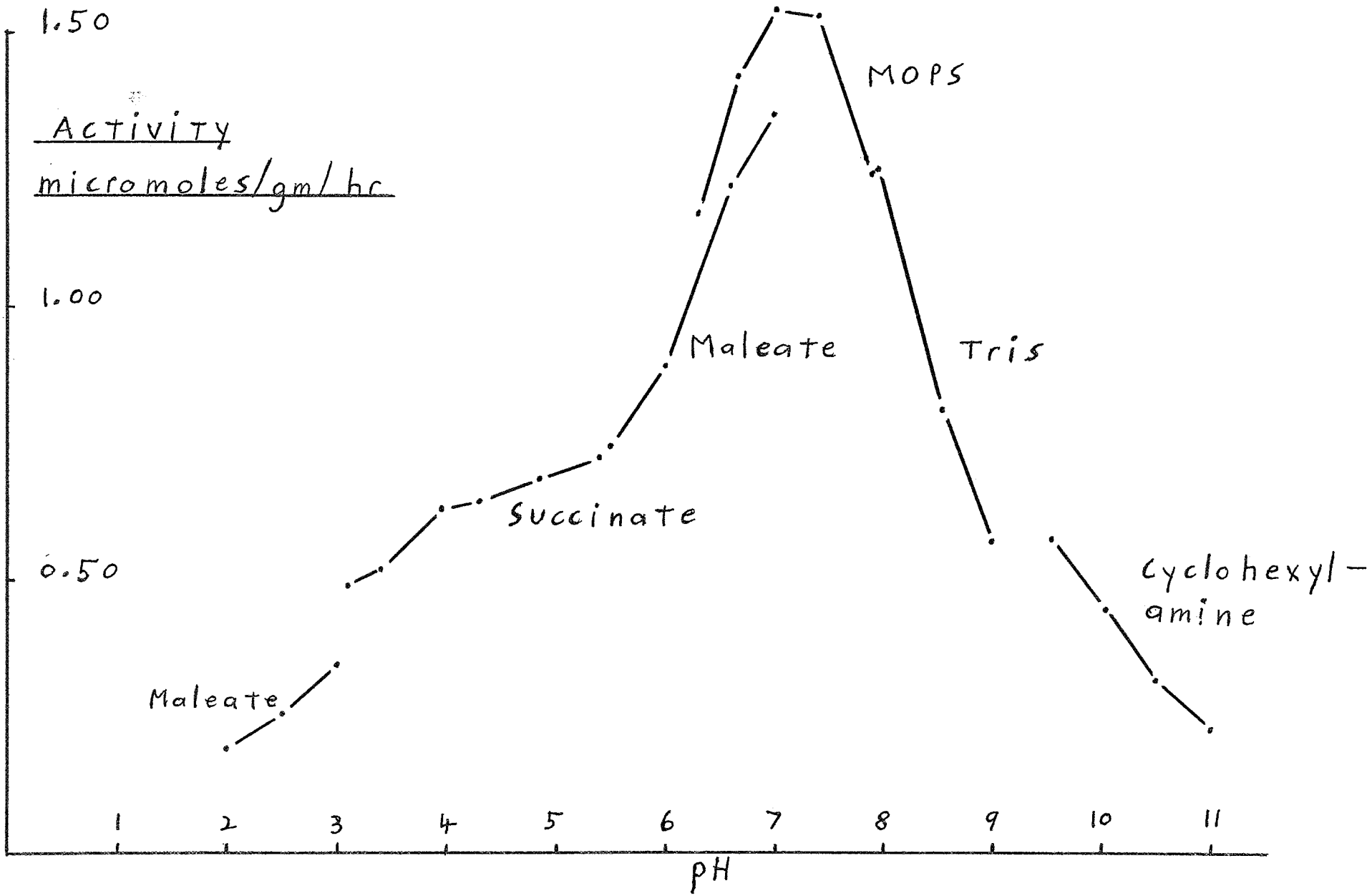
In place of the sodium maleate buffer, 4.0 ml of 0.10 M buffer of the appropriate compound was used. In some cases 0.1 M HCl or NaOH was added to bring the pH of the reaction mixtures and controls to the desired value. The buffers were prepared as 0.10 M stock solutions by neutralization with HCl or NaOH (I), (II). MOPS buffer was N-morpholino propane sulfonic acid obtained from Calbiochem, Los Angeles. The pka of this compound is 7.2. The concentration of substrate in the reaction tubes was 3.00×10^{-3} M. The reaction was allowed to proceed 1.0 hr at 25.0 °C with end-over-end agitation. The reaction was stopped by the addition of 2.0 ml of 1.0 M NaOH. All samples were prepared in duplicate and values for activity and pH are expressed as the average of duplicate tubes. The results of the experiment are shown in Figure 2.

In Figure 2 the phosphatase activity of New Dublin soil is plotted against the pH value of the reaction mixture. The data show that activity is detectable from pH 2.0 to 11.0. There is a peak in activity at a pH value of about 7.2 in 0.05 M MOPS buffer. Above and below this pH value the activity decreases. There is a "shoulder" in activity around pH 4.5.

The activity at the same pH value using different buffer compounds is not necessarily the same. For example, at pH 7.0 the phosphatase activity of New Dublin soil is 1.54 micromoles/gm/hr in 0.05 M MOPS buffer but 1.35 micromoles/gm/hr in 0.05 M maleate buffer. It appears

FIGURE 2

PHOSPHATASE ACTIVITY OF SOIL AT VARIOUS PH VALUES



that the different buffer compounds activate or inhibit the phosphatase reaction.

Buffers of citric acid were not used because it was found that these buffers extracted much colored matter from the soil, reducing the sensitivity of the colorimetric determination of the product of the phosphatase reaction.

The substrate appeared to be stable in the various buffer solutions over a pH range of 1.8 to 11.0; no variation in hydrolysis with pH was observed. In addition, the substrate appeared to be stable in alkali. The optical density of the alkalized substrate controls that had stood for four hours at 25.0 °C in the previous experiment (section C) was the same as that in the controls that had been analyzed immediately after the incubation period. The concentration of alkali in the control tubes was about 0.2 M NaOH.

E. Relationship between reaction rate for phosphatase activity and substrate concentration for New Dublin soil.

The relationship between the reaction rate for phosphatase activity of New Dublin soil in suspension and the substrate concentration was investigated. The phosphatase activity of New Dublin soil in suspension was determined at various concentrations of substrate from 1.00×10^{-4} M to 3.00×10^{-3} M. The reaction mixtures were buffered at pH 6.90 by 0.01 M sodium maleate buffer. The reaction was allowed to proceed for 1.0 hr at 25.0 °C,

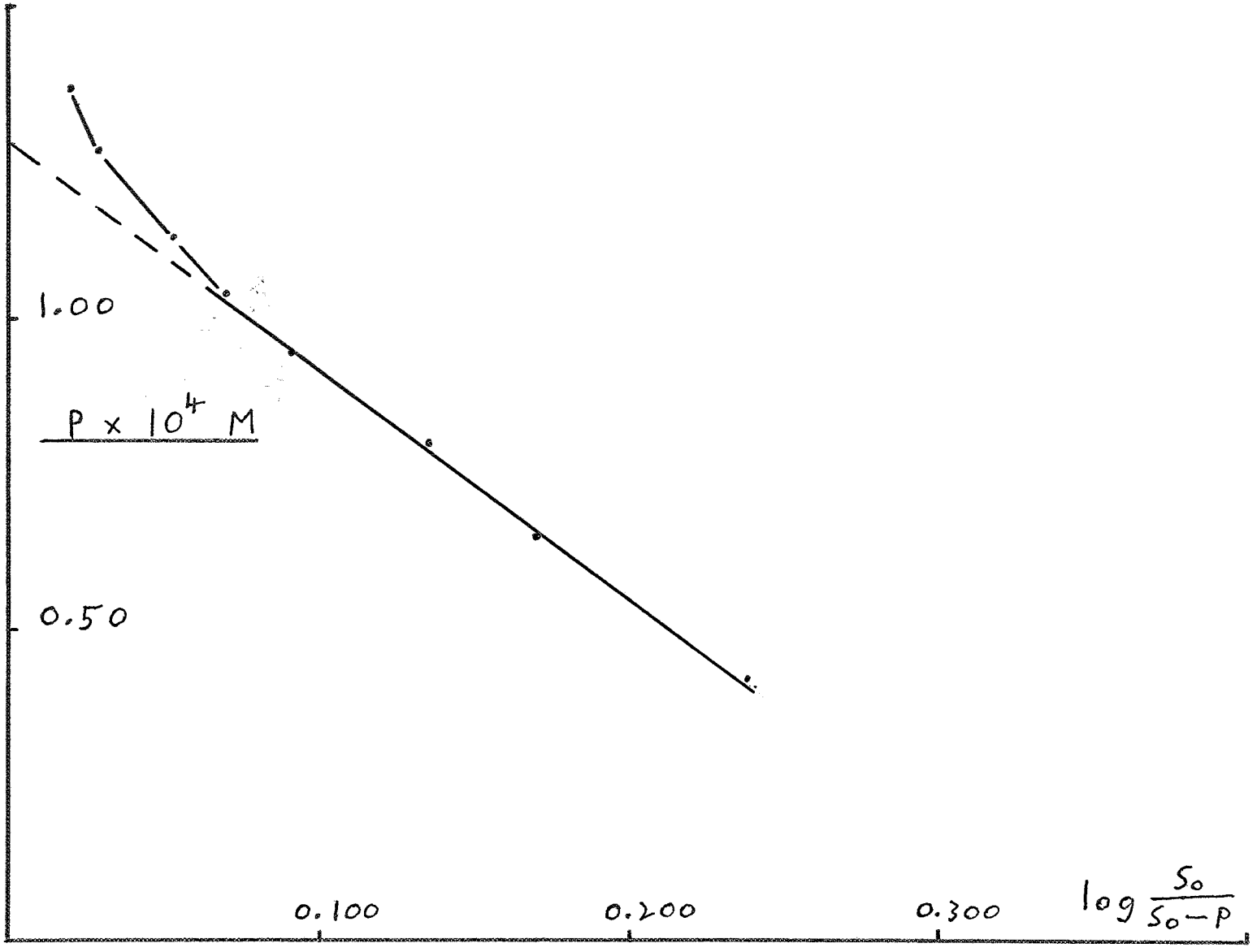
with end-over-end agitation of the tubes. All tubes were prepared in quadruplicate and the values for the concentration of product in the reaction tubes were expressed as the average of the quadruplicate samples.

The results of the experiment are shown in Figure 3. In Figure 3 the concentration of product, P, was plotted against log So/(So-P) where So is the substrate concentration, as described previously (3).

The curve relating the product concentration, P, to log So/(So-P) was linear for substrate concentrations from 1.00×10^{-4} M to 7.00×10^{-4} M but the curve bent progressively upwards at higher substrate concentrations (Figure 3). A linear relation between P and log So/(So-P) indicates that the reaction rate follows a Michaelis-Menten relationship to the substrate concentration (3). The data in Figure 3 indicate, therefore, that the reaction rate for phosphatase activity displayed by New Dublin soil in suspension at pH 6.90 in sodium maleate buffer followed a Michaelis-Menten relationship to the substrate concentration. This relationship held for substrate concentrations of 1.00×10^{-4} M to 7.00×10^{-4} M but not at higher concentrations. The value for Vmax calculated by extrapolation of the linear portion of the curve was 1.28 micromoles/gm/hr. The value for km calculated from the linear portion of the curve was 1.60×10^{-4} M.

FIGURE 3

PHOSPHATASE ACTIVITY OF SOIL: RELATION OF REACTION RATE TO SUBSTRATE CONCENTRATION



F. Hydrolysis of buffered solutions of para-nitrophenol phosphate by columns of soil crumbs.

Columns of crumbs prepared from New Dublin soil were perfused with buffered solutions of substrate and the hydrolysis of the substrate as a function of the substrate concentration and the flow rate was determined.

The columns were prepared in 0.01 M sodium maleate buffer pH 6.90. The columns contained 20 grams of crumbs and measured 2.2 cm in diameter by 8.3 cm high. The void volume was 25 cm^3. The temperature of the columns was maintained at 25.0 ^oC by a temperature-controlled water jacket surrounding the columns. The columns were perfused with substrate in 0.01 M sodium maleate buffer pH 6.90 at flow rates of 9.46, 2.80, and 1.48 ml/min. The corresponding average velocities of the solution through the columns are 3.14, 0.930, and 0.492 cm/min, respectively*.
The concentration of substrate used varied from 1.00×10^{-4} M to 3.00×10^{-3} M. The effluent of the columns was collected in samples of 25 ml. 0.5 ml of 0.1 M tetra-sodium ethylene diamine tetra-acetate and 0.5 ml of 1.0 M NaOH were added to each sample, and the concentration of product was determined by colorimetry (3). When the concentration of product in the effluent became constant with continuing perfusion, the value for the product concentration was taken as a characteristic of the column under the particular conditions used.

* velocity = column height/(void volume/flow rate).

The results of the perfusion experiments are shown in Table 3 and Figures 4, 5, and 6. In the Figures, the phosphatase activity of the column in micromoles/gm/hr is plotted against the concentration of substrate in the influent solution. Also, the concentration of product in the effluent is plotted against log So/(So-P), as before.

The data show that the phosphatase activity of the columns of soil crumbs increased with substrate concentration in a fashion similar to that for the soil in suspension. The activity approached a constant value at a substrate concentration of about 3.00×10^{-3} M. At the same values of substrate concentration, the activity of the columns was about 25% lower than that of the soil in suspension. The lower activity of the columns is undoubtedly due to the fact that material at the interior of the crumbs cannot participate in the phosphatase reaction.

At each value of substrate concentration the activity of the columns is lower, the lower the flow rate. Part of this effect is due to the fact that, because the phosphatase reaction hydrolyses the substrate, the concentration of substrate in the column is decreased, the decrease being greater the longer a unit of solution remains in the column, i.e. the lower the flow rate. Since the activity falls with decreasing substrate concentration, the lowered concentration of substrate in the column results in a lessened activity.

The decrease in activity with the lower flow rates

FIGURE 4

COLUMNS OF SOIL CRUMBS PERFUSED WITH SUBSTRATE: PHOSPHATASE ACTIVITY AND RELATION OF REACTION RATE TO SUBSTRATE CONCENTRATION

FLOW RATE = 9.46 ML/MIN

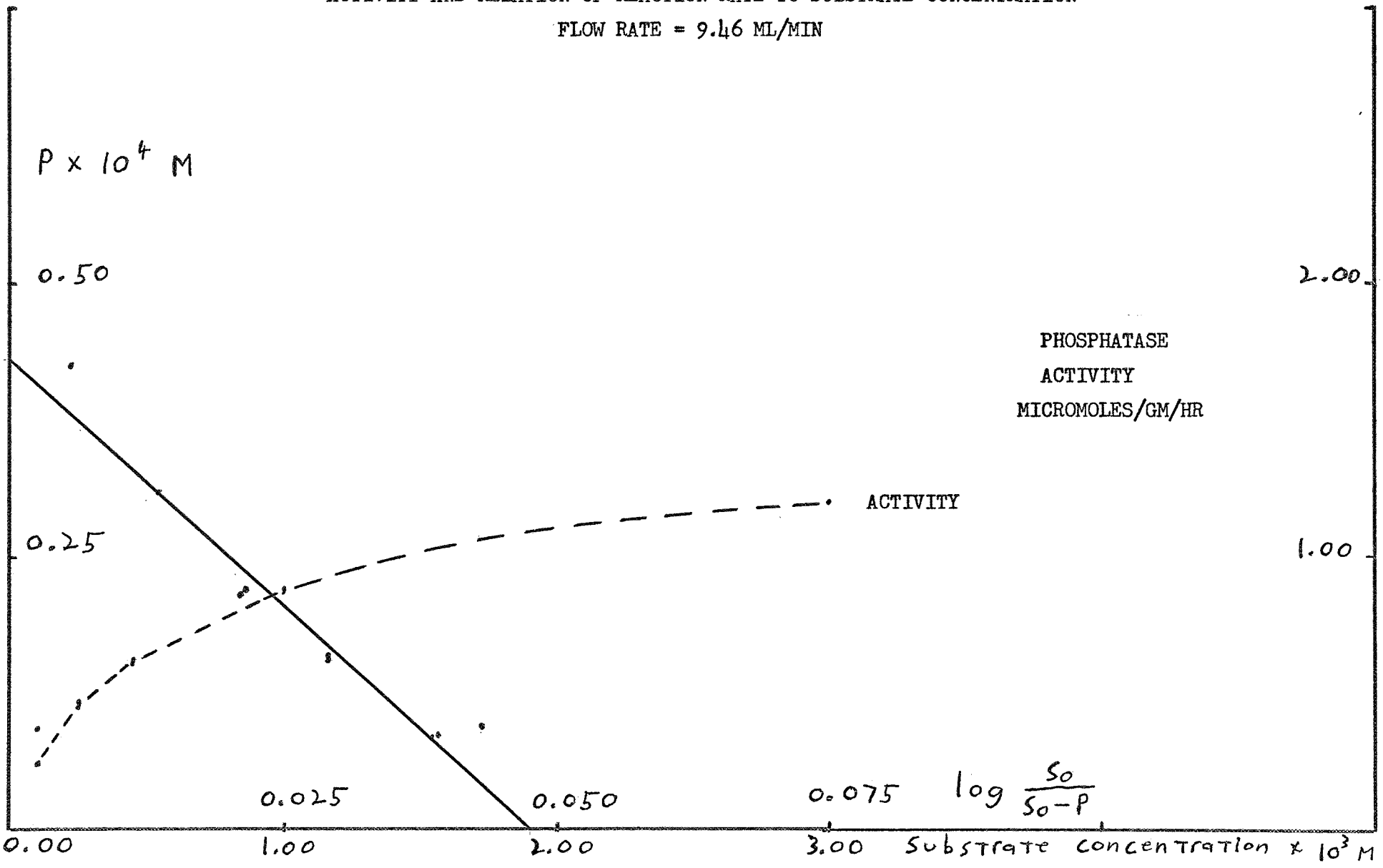


FIGURE 5

COLUMNS OF SOIL CRUMBS PERFUSED WITH SUBSTRATE: PHOSPHATASE ACTIVITY AND RELATION OF REACTION RATE TO SUBSTRATE CONCENTRATION

FLOW RATE = 2.80 ML/MIN

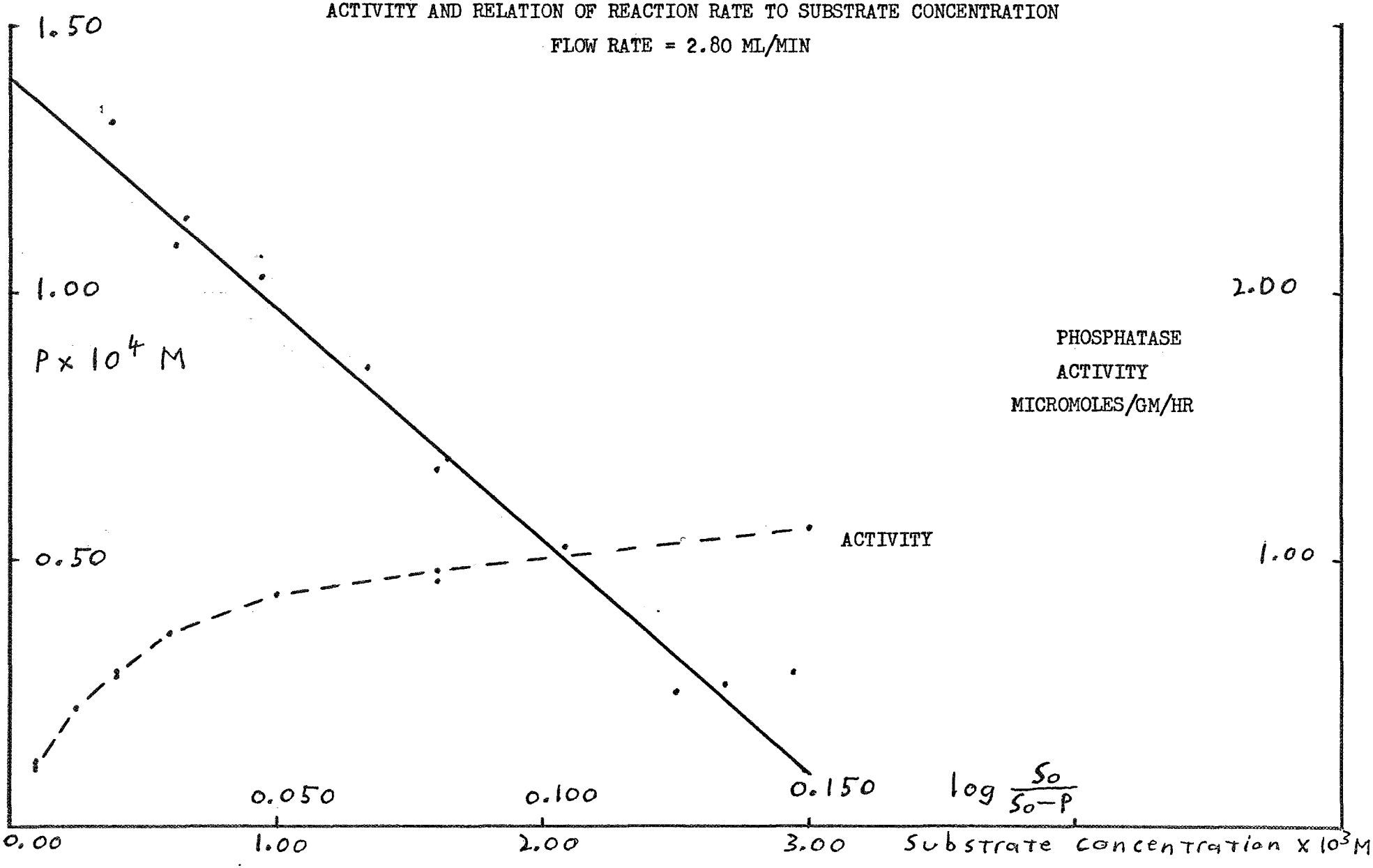
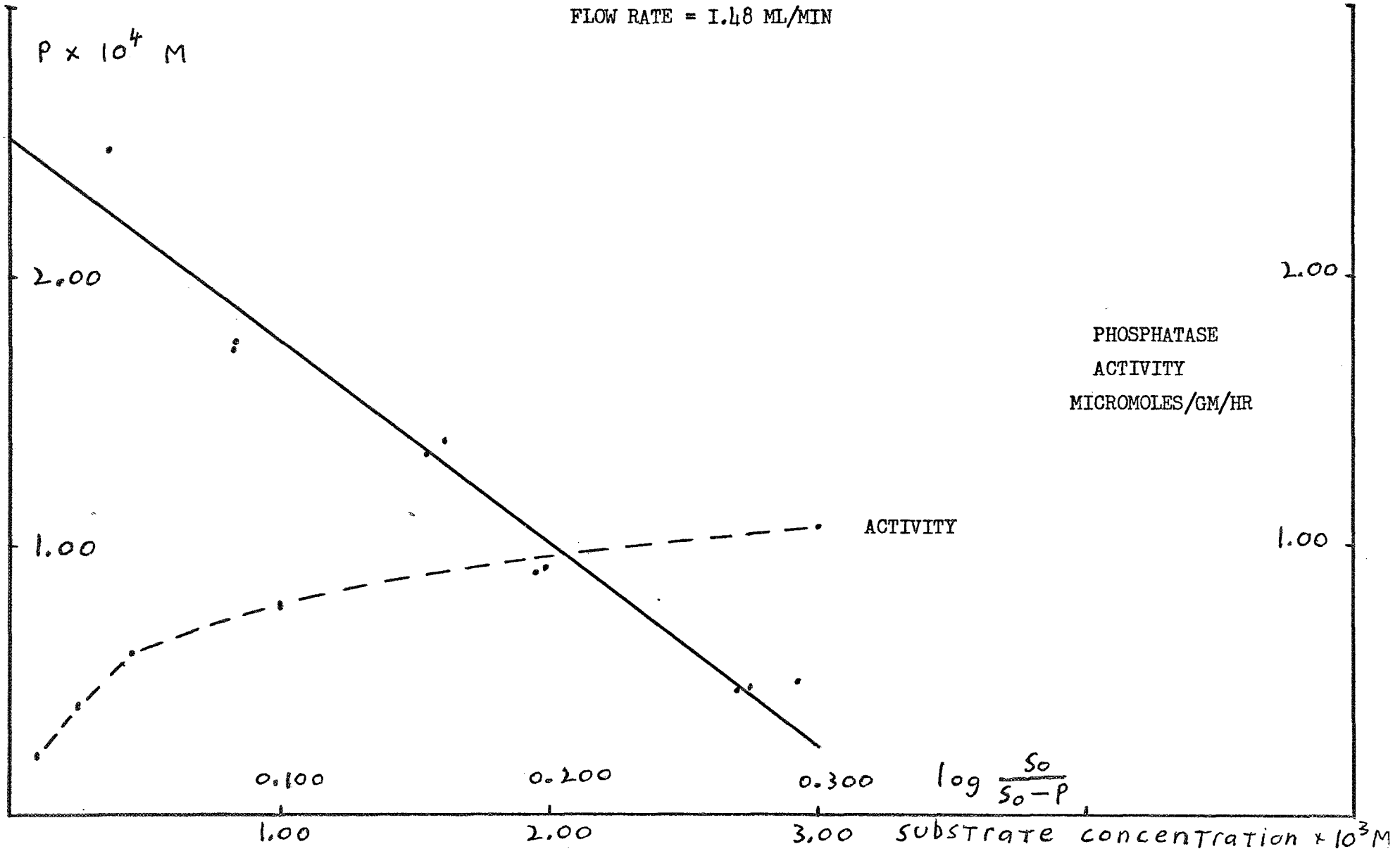


FIGURE 6

COLUMNS OF SOIL CRUMBS PERFUSED WITH SUBSTRATE: PHOSPHATASE ACTIVITY AND RELATION OF REACTION RATE TO SUBSTRATE CONCENTRATION

FLOW RATE = 1.48 ML/MIN



was still observed at high values of substrate concentration, under which conditions activity is relatively independent of small changes in the concentration. At 3.00×10^{-3} M substrate concentration the values for activity were 1.21, 1.12, and 1.07 micromoles/gm/hr at the flow rates of 9.46, 2.80, and 1.48 ml/min, respectively. However, these differences may reflect random experimental variation since these activity values are the data of one determination at each of the three flow rates.

In a separate experiment the phosphatase activity of the soil crumbs was determined following the method used for determining the activity of the soil in suspension. The activity was determined under the standard conditions given in section A of this report.

The average activity of five samples of crumbs determined in this way was 1.04 ± 0.07 micromoles/gm/hr. This value is in reasonably good agreement with the values for activity of the columns at the same concentration of substrate indicating the reaction occuring in the columns is similar to that for the crumbs in the reaction tubes.

The curves relating P, the concentration of product in the column effluents, to log So/(So-P) appeared to be linear within the range of substrate concentrations used (Figures 4-6). The deviation from the straight-line relationship observed for the soil in suspension at substrate concentrations above 7.00×10^{-4} M was not observed here. The results indicate, therefore, that the reaction rate for phosphatase activity of the columns followed a Michaelis-Menten relationship with substrate

concentration over a range of concentration from 1.00×10^{-4} M to 3.00×10^{-4} M.

The values for Vmax and km calculated from the curves are shown in Table 3.

Table 3. Michaelis-Menten constants for phosphatase activity of columns of soil crumbs perfused with buffered solutions of substrate.

flow rate ml/min	km ($\times 10^{-4}$ M)	Vmax umoles/gm/hr
1.48	3.30	1.12
2.80	3.78	1.17
9.46	3.94	1.22

The values of Vmax and km for the columns cannot easily be compared with those of the soil in suspension because the soil displayed Michaelis-Menten kinetics only over a more limited range of substrate concentration. However, the smaller value of Vmax for the columns compared to that for the soil undoubtedly arises from the fact that material at the interior of the crumbs cannot participate in the phosphatase reaction. The larger values for km for the columns compared to that for the soil in suspension may result from the fact that part of the reaction occurs in pockets formed by several adjacent crumbs or in pores in the crumbs themselves into which the substrate must diffuse in order for reaction to take place. The rate of

diffusion may limit the rate of reaction. A higher concentration of substrate would overcome this limitation, and the increase in concentration required would be reflected in a higher value of km (7), (8). Alternately, the Krilium present in the crumbs may alter the catalytic properties of the soil. The activity of enzymes attached to various support materials was greatly affected by the surface charge of such materials (4), (5), (9). If the support material had negatively charged groups on its surface and the substrate was also negatively charged, electrostatic repulsion would occur, lowering the effective concentration of substrate at the catalytic sites of the bound enzyme. An increase in the concentration of substrate would overcome this effect, the increase required being reflected in a value of km higher than that of the enzyme in a free state. Both Krilium (a synthetic polymeric poly-carboxylic acid) and the substrate would be negatively charged at the pH value of the columns (pH 6.90) and electrostatic repulsion may account for the higher value of km for the columns of crumbs compared to that of the soil in suspension.

The values for Vmax and km for the columns of soil crumbs increased with the flow rate (Table 3). An increase in Vmax with flow rate has been observed by others who used columns of enzymes attached to inert support materials (4), (5), (6), (7), (8), (9), (10). They attribute such increases to the presence of a film of stagnant liquid surrounding the surface of the material,

through which the substrate must diffuse in order to react. The film limits the rate of the reaction. As the flow rate increases, turbulence reduces the thickness of the film and the rate of the reaction increases. The disruption of the film by high flow rates also results in a decrease in the value for km. The <u>increase</u> in the value for km observed at the higher flow rates for the soil columns is more difficult to explain, but may be the result of a change in the pattern of flow of the substrate solution through the columns. Such an increase in the value of km with flow rate has been observed before (*8*).

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